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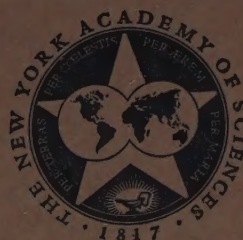
THE VAGINA

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WARREN R. LANG

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* This series of papers is the result of a conference on *The Vagina* held and supported by The New York Academy of Sciences on April 10 and 11, 1959.

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INTRODUCTORY REMARKS

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All branches of basic and clinical science require a periodic reassessment of past reported work in relation to more modern techniques and an effort to seek newer concepts for further channels of investigation. This we have tried to do. When new or unfamiliar methodologies are employed, the authors have attempted to orient us with respect to their aim and implementation before outlining results and their significance. Thus, it is hoped that this monograph will satisfy both the basic scientist and the clinician.

The intent of this publication is twofold: to collate the highlights of what is known of vaginal structure and function, and to offer a panoramic view of recent research in the field. Various aspects of embryology, physiology, biochemistry, microbiology, immunology, enzymology, endocrinology, and pathology have been considered. This monograph includes a group of papers on the ever-timely subject of vaginal infections and several papers related to reproduction. Each author is well qualified to discuss his topic. To keep the presentations on a more academic but no less practical plane, considerations of specific therapy have been omitted. It is hoped that in this way the monograph will fulfill a definite need for a concentrated, accurate, up-to-date source of postgraduate information on basic vaginal problems.

Part I. Vaginal Anatomy and Physiology

EMBRYOLOGY AND ANATOMY OF THE VAGINA

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Embryology

The embryology of the vagina is of special interest for several reasons, including the fact that carcinoma of the cervix uteri may originate in the epithelium of the vaginal portion of the cervix (portio vaginalis). Also, a common feature of "female pseudohermaphroditism" is absence of the lower portion of the vagina. The original vaginal epithelium is derived from the fused Müllerian ducts. It is replaced during prenatal life, however, by entoderm from the urogenital sinus and/or by ectoderm from the proctodeum.

As to the particulars, the Müllerian ducts develop in a monkey embryo of 36 days (Heuser and Streeter, 1941) and in human embryos of 37 days' ovulation age (Streeter, 1948). The first manifestations are bilateral thickenings of the mesothelium (coelomic epithelium) at the cephalic poles of the mesonephroi. The invagination of these thickenings produces the ostia. The growing tips of the ducts are solid (FIGURE 1). The ducts follow the Wolffian ducts in order to reach the cloaca. The evidence that the Wolffian ducts induce the formation of the Müllerian ducts by acting as embryonic organizers is threefold. First, the experimental destruction of the Wolffian duct in the chick embryo is followed by failure of the corresponding Müllerian duct to reach the cloaca (Grünwald, 1937). Second, absence of the human Müllerian duct is associated with congenital absence of the kidney (Homma, 1956), and renal agenesis in man is a consequence of retardation in development of the ureter, of embryonic agenesis of the ureter, or of arrest in development of the parent tissue of the ureter, for example, the Wolffian duct (Boyden, 1932). The third evidence is that in a young embryo the Müllerian and Wolffian ducts initially lack any intervening basement membrane (Grünwald, 1941).

The growing tips of the Müllerian ducts eventually reach the dorsal wall of the urogenital sinus (for example, ventral half of the cloaca). They are solid epithelial tips, and end blindly on the Müllerian tubercle (Hunter, 1930). In the pelvic region of the embryo the right and left Müllerian ducts cross the Wolffian ducts ventrally, approach each other in the midline, and begin to fuse with each other to produce the uterovaginal canal (Koff, 1933). This fusion progresses cranially. For some time the blind ends of the ducts are separate structures with an intervening mesenchymal septum (Meyer, 1937), but this feature is not depicted by Koff's figures B and C, Plate 1 (embryos 4304 and 4289, horizons XXI and XXIII, respectively, Streeter *et al.*, 1951).

The genitoinguinal ligament (FIGURE 1) is useful as a landmark for distinguishing the two derivatives of the uterovaginal canal, namely, uterus and vagina. It is the forerunner of the round ligament of the uterus in a female and of the gubernaculum testis in a male (FIGURE 2).

According to an early description that is not generally accepted in full today,

the upper four fifths of the human vagina originate from the uterovaginal canal and the lower one fifth from the urogenital sinus (Koff, 1933); bilateral primordia of the lower one fifth are described as dorsal evaginations of the urogenital sinus, and are called sinovaginal bulbs; a solid mass of epithelium derived in part from the uterovaginal canal and in part from the urogenital

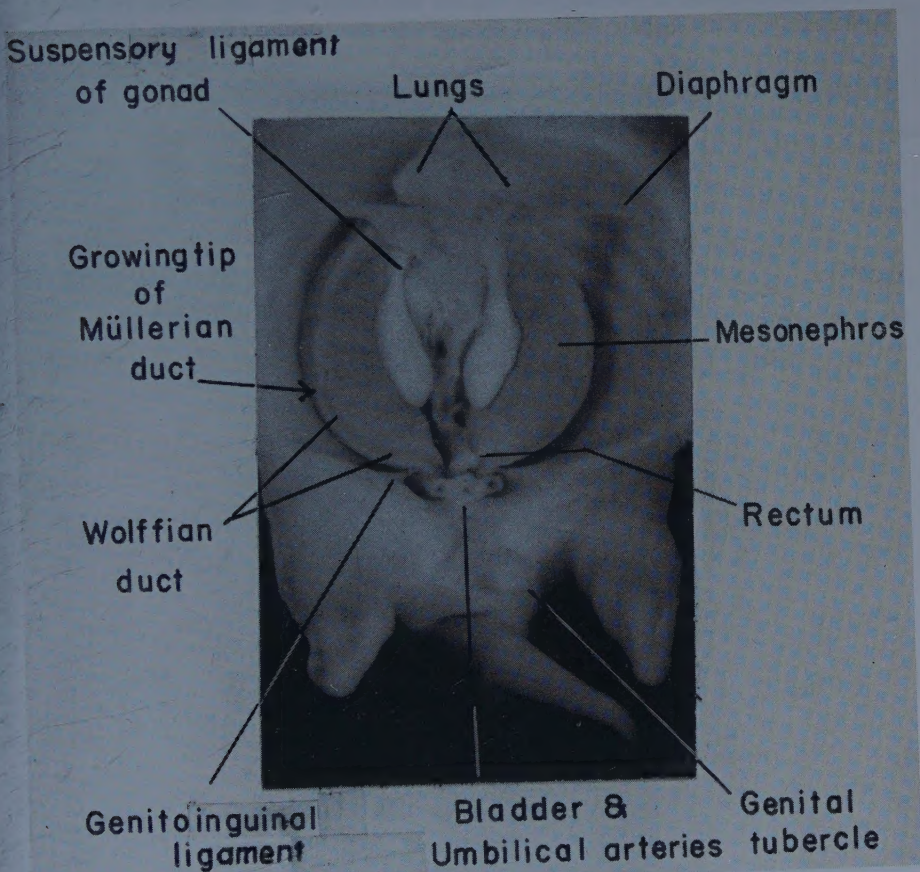


FIGURE 1. Ventral view of the Müllerian ducts in a bovine embryo of approximately 40 days, 22 mm. (crown-rump). The growing tips of the Müllerian ducts are solid epithelial structures that as yet have not reached the cloaca. Not illustrated is the fact that stained sections of the gonads showed primary sex cords. Author's preparation. $\times 9$.

sinus is designated the primitive vaginal plate. Certain elements of Koff's description (1933) have been superseded by the observation that eventually all the original vaginal epithelium disappears and is replaced by new epithelium which migrates cranially from the cloacal region.

The view that the definitive vaginal epithelium arises exclusively from the entoderm of the urogenital sinus was advanced by several investigators, notably, Erna Vilas and Robert Meyer. Vilas (1932), working in the Embryolo-

gisches Institut in Vienna, Austria, studied 25 fetuses ranging in crown-rump length from 32 to 200 mm. Meyer, at the Pathologisches Institut of the Universitäts-Frauenklinik in Berlin, Germany, published a series of 6 extensive papers on the development of the vagina, the first of which appeared in 1934; he summarized his careful and discerning observations in 1937.

The replacement of the original vaginal epithelium by the definitive epithelium is finished or largely finished by the 140-mm. stage (Bulmer, 1957), the

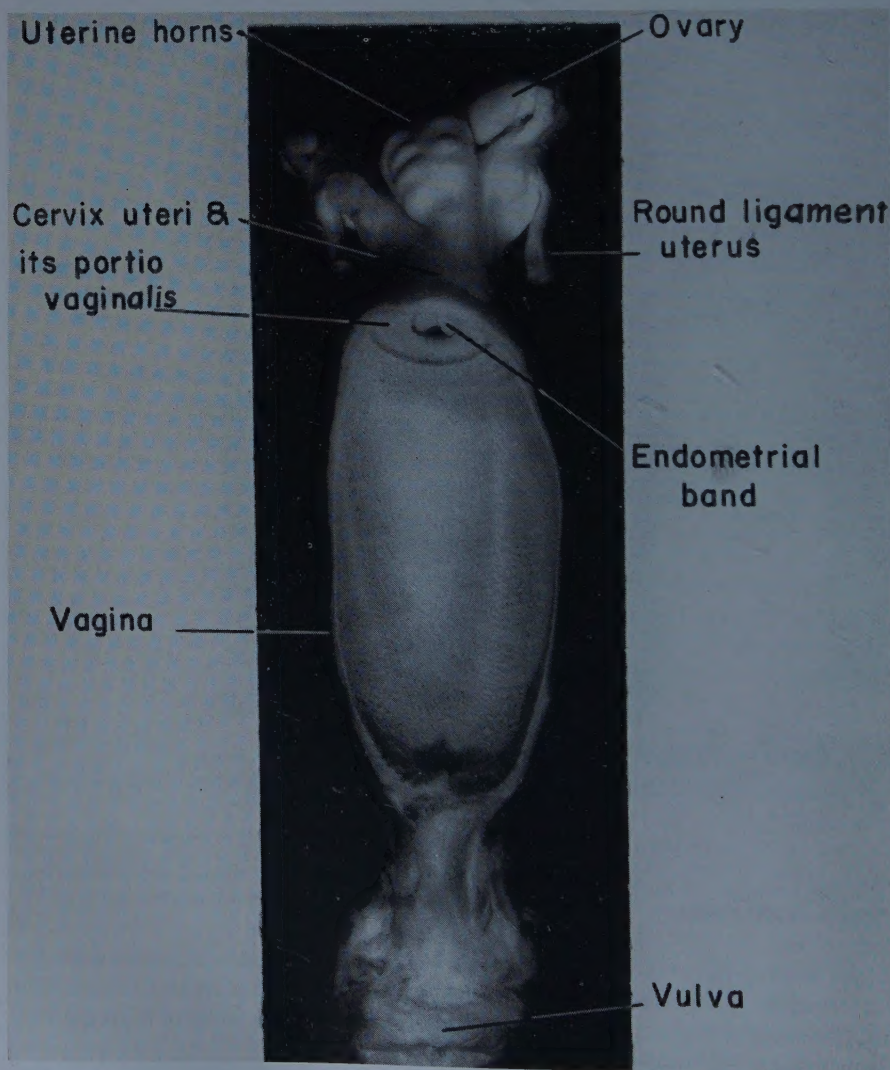


FIGURE 2. Dorsal view of vaginal cavity in a bovine fetus of about 150 days, 380 mm. (crown-rump). The dorsal wall of the vagina has been removed in order to expose the vaginal portion of the cervix uteri and certain endometrial bands (rugae) of the cervix. Author's preparation. $\times 2$.

stage illustrated in FIGURES 3 and 4. At an earlier stage of 135 mm., one author claims that the posterior fornix is the site of junction of the Müllerian epithelium and the definitive epithelium (Politzer, 1955).

The cellular responses to experimentally introduced estrogen led to the current view that the definitive vaginal epithelium is derived either entirely or in part from the ectoderm of the cloacal membrane and/or proctodeum and that it reaches the vagina via the urogenital sinus. In the opossum such epithelium migrates upward as far as the cervix uteri and the trigone of the bladder (Burns, 1942). In monkeys, the same phenomenon seems to occur

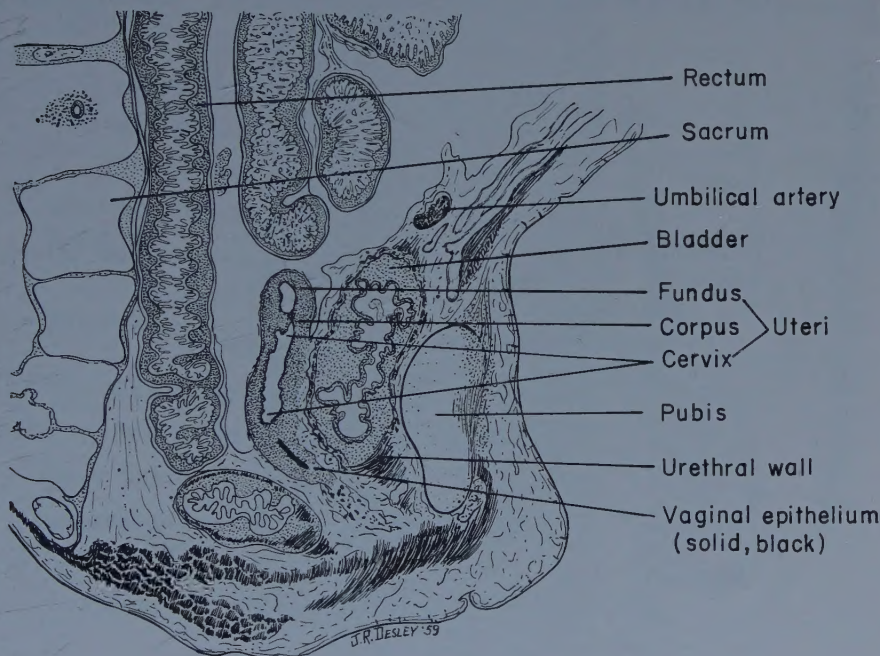


FIGURE 3. Sagittal section of part of the vagina in a human fetus of 140 mm. (crown-rump). The vaginal cavity has not appeared as yet. Author's preparation. $\times 4$.

(Zuckerman, 1940). In fact, there is no apparent line of histological demarkation separating the proliferative response of the vagina to estrogen from that of the vestibule of the vulva and, in certain species, from that of skin beyond the vulva, the so-called sex skin (Zuckerman, 1950).*

The final vaginal epithelium undermines the Müllerian epithelium, which in turn becomes eroded and disappears (Meyer, 1937; Burns, 1942). This phenomenon of erosion is known as pseudo-erosion congenita (Hunter, 1930).

All things considered, I think that Koff's (1933) sinovaginal bulbs are not discrete evaginations of the urogenital sinus. Instead, I believe that they are merely regions of junction of two kinds of epithelium, namely, the mesodermal

* Such upward migration of ectoderm from the cloacal region is in line with the fact that the anal canal is lined by squamous epithelium.

epithelium of the right and left Müllerian ducts and the entodermal epithelium of the urogenital sinus. The basis of my belief is twofold: (1) the right and left Müllerian ducts retain their separate junctions with the urogenital sinus for some time after the uterovaginal canal has been formed at a pelvic level superior to the Müllerian tubercle (Meyer, 1937); (2) they also do this in the male rat fetus (Wells *et al.*, 1954).

Experimental Hermaphroditism

Although the literature contains many observations on this subject in infra-primates, we shall deal only with experiments in monkeys. When sufficient

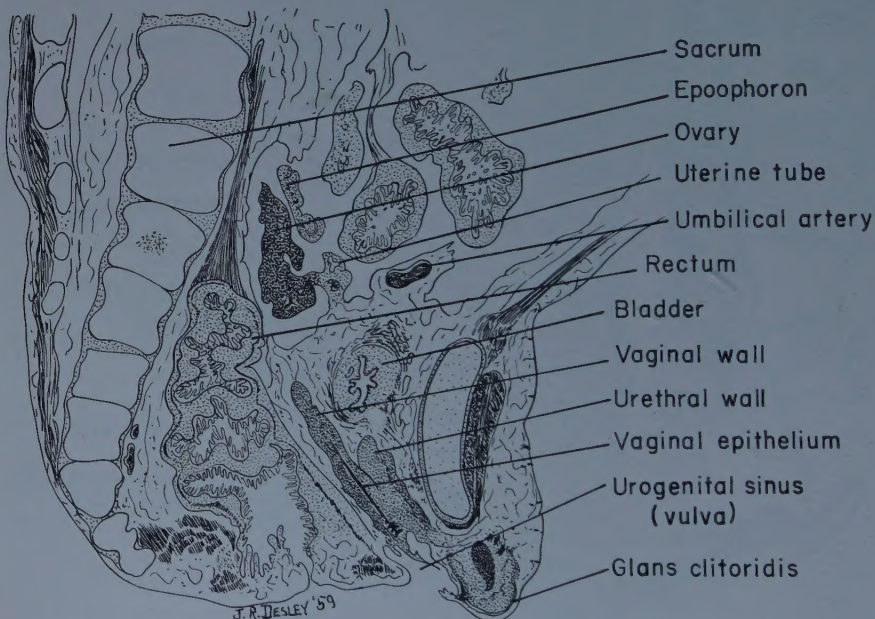


FIGURE 4. Another section of the sagittal series from which FIGURE 3 was obtained; human fetus of 140 mm. (crown-rump). Author's preparation. $\times 4$.

androgen is introduced into the circulation of a female fetus during the appropriate stage of development, the induced abnormalities include absence of the lower portion of the vagina. Two methods of introducing the androgen have been used. First, it was given to the gravid female by intramuscular injections (van Wagenen and Hamilton, 1943; Wells and van Wagenen, 1954). Second, it was injected into the amniotic sac (Dantchakoff, 1950).

The induced agenesis of the lower part of the vagina involves an abnormal persistence of the urogenital sinus and the subsequent elongation of it (FIGURE 5). This persisting sinus simulates somewhat the urethra of a male in that it is as long as the elongated phallus. It subserves, however, the voiding of urine and the passage of menstrual blood (van Wagenen, 1949; Wells and van Wagenen, 1954).*

* The introduced androgen is not necessarily a specific inhibitor of the formation of the

Fully Formed Vagina

It was decided to present only a brief account of the fully formed vagina, especially since this subject has been considered recently in extensive reviews of the morphology and physiology of the reproductive tract (Eckstein and Zuckerman, 1956*a, b*).

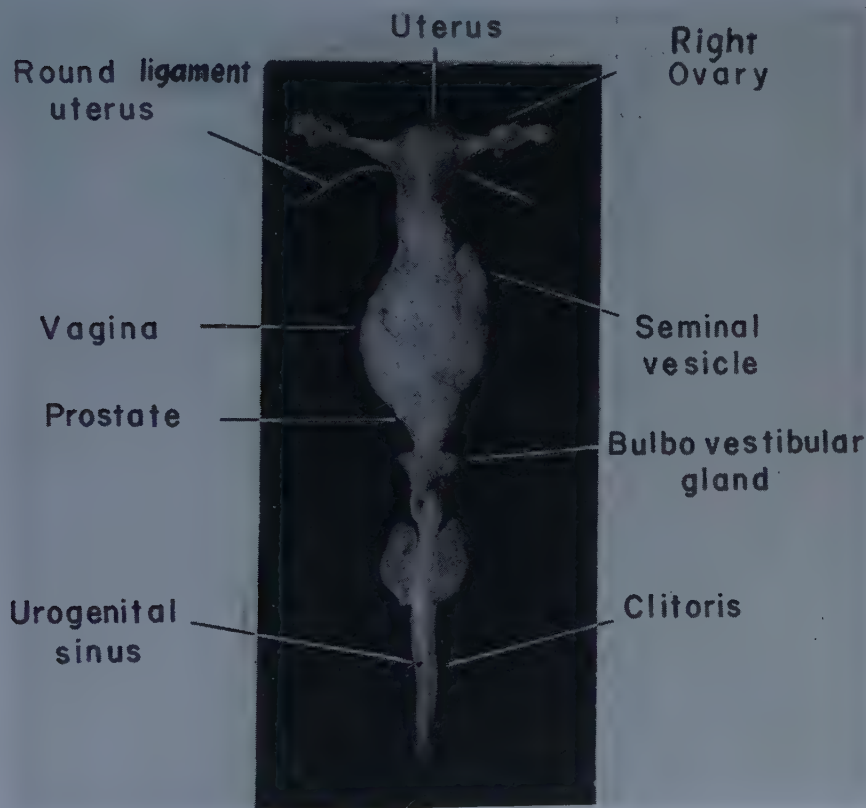


FIGURE 5. Dorsal aspect of the reproductive tract in a monkey of 8 years in which female pseudohermaphroditism had been induced experimentally by giving its mother 25 mg. of testosterone propionate/day from day 37 of gestation to day 64, a total of 665 mg. The monkey showed agenesis of the lower portion of the vagina and an abnormal persistence of the urogenital sinus.

From literature considered in a more recent review (Wells, 1959) it is known that the epithelial cells of the human vagina are bound together by protoplasmic bridges. When the cells are studied by electron microscopy, they show cytoplasmic fibrillae that run from cell to cell (Bahr and Moberger, 1956).

In the vagina of the rat, the content of alkaline phosphatase varies with the

lower segment of the vagina; it is known, for example, that the subsection of pregnant rats to vitamin A deficiency leads to many congenital anomalies in the offspring, including the anomaly of agenesis of the lower vagina (Wilson and Warkany, 1948).

ovarian cycle (Ford, 1956). Two peaks occur, one during proestrus and one during metestrus.

Other studies deal with the rugae in the human vagina (Orsós, 1957), with the acidity of the mammalian vagina (Lang, 1955), and with the results of

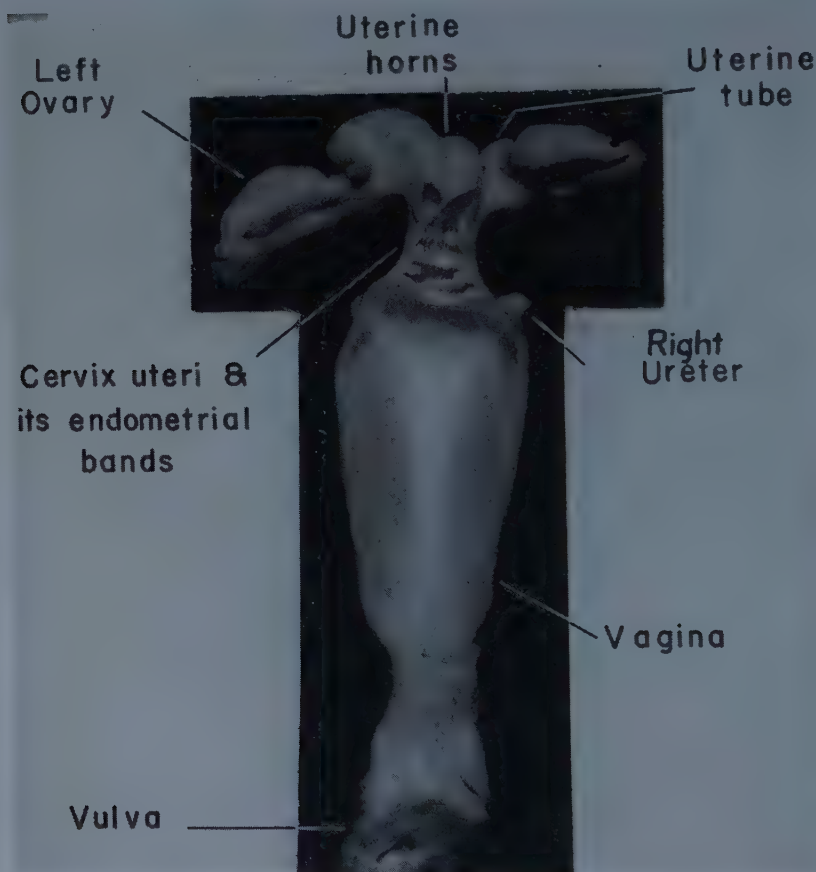


FIGURE 6. Dorsal view of vaginal cavity in a bovine fetus of about 100 days, 226 mm (crown-rump). The dorsal wall of the vagina and that of the uterine cervix have been removed in order to show the endometrial bands (rugae) of the cervix. Author's preparation. $\times 2$.

locally applied vitamin A and estrogen upon the vagina of the ovariectomized rat (Kahn, 1954).

In bovine fetuses, the vaginal communication with the uterus is guarded by endometrial bands that are situated in the cervix uteri (FIGURES 2 and 6).

The growth of the human vagina during prenatal life reflects the action of the hormones of pregnancy. The sharp dip in the growth curve during early postnatal life reflects the lack of hormones of pregnancy.

The relatively large size of the vagina at term may be correlated with the fact that in the newborn the empty bladder extends well above the symphysis

pubis. Only later, when the bladder lies opposite the symphysis, does the uterus show its definitive antelexion and anteversion (FIGURES 3 and 4).

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THE GROSS AND MICROSCOPIC ANATOMY OF THE HUMAN VAGINA*

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The surgical approach to the anatomy of the vagina has been discussed at length by many authors; however, there is a dearth of pure anatomical studies. This paper reviews the literature and contributes our findings to the various observations on the gross and microscopic anatomy of the vagina.

Gross Anatomy

The term vagina, given to the organ by Colombo¹² was taken from the Latin, meaning sheath. The vagina serves as the intermediary or connecting organ between the external genitalia and the uterus. In the adult it varies in length from 7 to 10 cm.; the posterior wall is approximately 1.5 to 2 cm. longer. The topographical landmarks are notably the rugae vaginales,¹² which are folds of the vagina that pass laterally upward and posteriorly on either side toward the midline and connect to a longitudinal ridge in the midline known as the column rugarum.^{30, 52, 74} In the area of the urethra this ridge becomes more prominent and is termed the urethral carina. In the region of the origin of the urethral carina a deep lateral groove and fold are present. This, in conjunction with a similar fold less prominent in the posterior vaginal wall at the upper level of the levator ani, has become known as the fold of Shaw.

The anterior vaginal wall^{70, 71} terminates in a circular fissure around the cervix uteri where the cervix projects into the vagina. The groove or space thus formed between the cervix and the anterior vaginal wall is termed anterior fornix. Similarly, the larger space formed posteriorly is termed the posterior fornix. The vaginal opening is partially covered by a membrane, the hymen. Depending upon age, parity, and sexual activity it will vary in shape and size. When it is ruptured, the small tags of tissue remaining are termed curunculae hymenales. According to most anatomists, the transverse section of the undistended vagina resembles the letter H. In my studies I have found the shape to vary from stellate, just within the hymenal ring, to H shape, under the urethra and middle third, to a crescent shape, just prior to the cervix uteri. In older specimens where atrophy has occurred the organ may be crescent-shaped in its entirety.

The gross relationships of the vagina to surrounding organs have been variously described. According to one investigator,²⁰ the vagina in the nulliparous reclining woman assumes a downward direction of 55° to the vertical in its lower two fifths, 10° in the second two fifths, and approximately 55° in the last fifth. The over-all angle is 30°. These changes of direction cause the vagina to take the shape of an S or italic letter F curve as it descends into the pelvis. The long axis of the vagina forms an angle of 90° with that of the normal ante-

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† Markle Scholar in Medical Science.

verted uterus.¹⁶ The cervix uteri, therefore, is directly opposite to the posterior vaginal wall. Anteriorly,^{18, 30, 56, 74} the vagina is intimately related to the urinary vesicae or bladder from the cervix to the symphysis pubis, at which point the urethra emerges to course approximately one third of its length on the vagina, similar to that of the urinary bladder. The urethra then enters into the vaginal wall to become an inseparable part of it, and finally terminates with its external meatus at the introitus. In the region of the upper third of the urethra and at its junction with the bladder, heavy bands of tissue pass from the anterolateral walls of the vagina, in conjunction with similar fibers from the urethra and vesical neck, to insert themselves into the rami of the pubic symphysis.^{5, 41, 53} These have been termed the puboprostatic ligaments of the female.⁴¹ Many authors have described a thick connective tissue layer interposed between the vagina and bladder and upper third of the urethra, which has variously been termed pubocervical fascia^{24, 38} and "musculofascial sheet."⁶ Other investigators⁷⁸ have felt that this layer was most probably synonymous with the puboprostatic ligaments.⁴¹ In most of the recent studies^{29, 41, 63} the absence of a fascia is reported; instead, the presence of a loose connective tissue adventitia of the vagina, having no supporting capacity, is described. The ureters may be observed on either side in the region of the upper third of the vagina as they enter the muscularis of the bladder. They are found coursing in an oblique direction along the anterolateral margin of the vesicovaginal junction, and actually exist as separate entities between the bladder and the anterior vaginal wall. Posteriorly, the upper fourth of the vagina is related to the rectouterine space, the cul-de-sac described by Douglas,^{11, 23} which is covered with peritoneum. The middle half of the vagina is in close relationship to the rectum, only the adventitia of both organs being interposed. In the lower fourth of the vagina, the anal canal has begun and the two organs are separated by the sphincters of the anus and rectum, as well as the interposing perineal body containing the origin of the bulbocavernous and superficial transverse perineal muscles. In the region of the hymenal ring the vagina is covered on its lateral parts by the bulbocavernous muscles and bodies. The fact that the muscle invests the vestibular bulbs or cavernous bodies is the reason that it long has been termed the sphincter vaginae.

The blood supply of the vagina is unique in that it has sufficient cruciate anastomoses to allow it adequate blood supply in the event of injury to any of its routes of supply. Beginning superiorly, the blood supply originates with the uterine, which gives off a descending branch, the cervicovaginal artery. This artery corresponds to the inferior vesical artery of the male. Several branches go to the cervix, the most prominent anastomosing with that of the opposite side to form the coronary artery of the cervix. The vaginal arteries branch on the lateral aspect of the vagina to send rami to both the anterior and posterior surfaces of the organ. It should be noted that not infrequently branches of the internal iliacs, known as arteriovaginalis, similarly may supply this region. When this occurs, the inferior vesical artery supplies the middle half of the vagina. The lower half of the vagina is supplied by ascending branches of the middle hemorrhoidal arteries. The latter divide to send rami to the anterior, as well as posterior, walls of the vagina. The various rami of the arteries supplying the vagina⁷⁵ (both right and left sides) meet in the

midline on the anterior, as well as posterior surfaces, to form a single median vessel, the azygos artery of the vagina.³⁹ The final anastomosis occurs between the urethral branch of the dorsal artery of the clitoris which originates from the internal pudendal artery and the vaginal branches of the middle hemorrhoidal vessels. The azygos artery terminates just prior to the point at which the urethra enters the anterior vaginal wall. Drainage of the venous system of the vagina is consummated through a series of plexuses. In the lower portion of the vagina the drainage is along the urethra onto the perineum and into the dorsal vein of the clitoris, as well as into the middle hemorrhoidal venous plexus. The greater part is perineal in direction (internal pudendal plexus), terminating in the internal pudendal vein. This vein may be single or paired, and communicates directly with the hypogastric veins of its side. The upper vaginal venous plexus joins the uterine and cervical veins to drain into the hypogastric veins.

The lymphatics of the vagina are related closely to those of the adjoining organs.⁶⁵ They begin with an easily demonstrable mucosal plexus⁶⁰ and drain into a deeper muscular network. The collecting trunks observed in my own studies, as well as those noted by others,^{8, 10, 60, 65} form an indefinite pattern. The muscular plexus forms an irregular network on the anterior and posterior surfaces of the vagina with anastomoses between both sides. This multitude of small lymphatics coalesces into larger channels, usually two to four in number. There is some disagreement among authors as to the direction of flow along the lateral walls of the vagina. Many of them believe that the trunks drain into three distinct directions: superior, middle, and inferior.⁶⁰ The superior group of lymphatics join those of the cervix, while the middle group of collecting vessels course along the vaginal arteries toward their origin. The superior group of lymphatics follow the cervical vessels to the uterine artery, where they pass the ureter both superiorly and inferiorly¹⁰ and accompany the uterine artery to terminate in the medial chain of the external iliac nodes. Occasionally they have been observed to enter the node of the obturator foramen.¹⁰ The middle group, which drains the greater part of the vagina, terminates in a hypogastric node at the origin of the vaginal artery. It is not infrequent to observe small nodes along their course.^{8, 52, 60} In addition, my observations confirm the presence of intercalating nodules on the posterior vaginal wall in the rectovaginal septum.^{10, 52} The third, or inferior group of lymphatics, supplies the area around the hymen. These vessels form frequent anastomoses between right and left sides and course in two distinct directions in the adult.⁶⁰ In part they course upward to anastomose with the middle group of the vagina to terminate in the respective pelvic nodes; the remaining channels enter upon the vulva to drain into the respective group of inguinal nodes. The anastomoses of the vaginal lymphatics are many. Superiorly, they join readily with those of the uterus and cervix and, in the middle and inferior area, with the vulva, urethra, and rectum. The vaginourethra-vesical lymphatic relationship has been one of conjecture.⁶¹ My observations confirm a separation between vesical and vaginal drainage; however, in the region of the lower two thirds of the urethra this separation is not as clear. Because of the intimate relationship of the urethra with the anterior vaginal wall I feel that the urethra joins, at least in part, the vaginal lymphatics (inferior group)

to drain onto the vulva. There is no doubt that in the lower third of the vagina connections do occur between the posterior vaginal wall and the rectum.^{10, 55} The drainage, however, is not maximal along this route, which follows that of the rectal stalk. It should be noted that the pararectal nodes are distinctly different from those of the previously described paravaginal chain.⁵² There has been considerable discussion of the flow of lymph into the vagina from the vulva and possible retrograde flow from the hymenal area.^{10, 60, 62, 65} The present evidence leads me to believe that this does occur, but not by preference. The uterovaginal nodes of Sappey (hypogastric nodes of vaginal arteries at the junction of vaginal arteries, ureter, and uterine arteries), as well as the nodes in the base of the broad ligament and at the uterovaginal wall,^{8, 46} may be considered intercalated nodes and are inconstant in number and position.

The gross innervation of the vagina⁴¹ has been studied, for the most part, with that of the uterus. The pelvic autonomic system originates with the superior hypogastric plexus.^{15, 41, 47} The middle hypogastric plexus^{15, 26, 41} passes into the pelvis to the immediate left of the midline.^{32, 41} At the level of the first sacral vertebra the plexus divides into branches going to the right and left sides of the pelvis. These branches form the beginning of the inferior hypogastric plexus.^{15, 19, 26, 43} The inferior hypogastric plexus is therefore a divided continuation of the middle hypogastric plexus,^{26, 41} the superior hypogastric plexus,²⁷ the presacral nerve,^{27, 49} or the uterinus magnus.¹⁹ This group of nerves descends into the pelvis in a position posterior to the common iliac artery and anterior to the sacral plexus, curves laterally and finally enters the sacrouterine folds or ligament.^{15, 19, 26, 41, 43} The medial segment of the primary division of the sacral nerves ($S_2 S_3$,⁴⁷ $S_2 S_3 S_4$,⁴⁹ $S_1 S_2 S_3 S_4$ [?] S_5 ,⁴³ and $S_2 S_3 S_4$ [?] S_6)²⁶ send fibers (nervi erigentes) into the pelvic plexus within the sacrouterine folds.^{19, 26, 27, 41, 53} Therefore, the plexus appears to contain both sympathetic (inferior hypogastric plexus) and parasympathetic (nervi erigentes) components.⁴¹ Within the base of the broad ligament an extension of this plexus contains many ganglia.^{15, 19, 35-37, 41} The ureter occupies a position superficial to the ganglia.²⁶ The plexus is supplied by the middle vesical artery.²⁶ No specific pattern of the supply to each adjacent organ has been reported as yet.^{26, 37, 41, 58} The greater number of nerves appears to enter the

PLATE I.

a. A section through the introitus demonstrating the close relationship between the urethra, anterior vaginal wall, bulbocavernosus muscle, and the cavernosus bodies. The inseparability of the urethra and anterior vaginal wall is demonstrable. In addition, the decussating fibers of the bulbocavernosus muscles that will form, in part, the striated sphincter of the urethra can be observed on the superior surface of the urethra. Note the many folds in the vagina and the para-periurethral glands.

b. A section of the gland through the middle and lower third of the vagina demonstrating the pubococcygeus muscles, the bulbocavernosus muscles, the urethra with its striated sphincter, the vagina with its many folds, and the rectum. Note the close relationship between the urethra, vagina, and rectum.

c. A higher power photomicrograph of *b* demonstrating the striated sphincter of the urethra and its smooth circular and longitudinal muscle, the musculature of the vagina and the bulbocavernosus ischiocavernosus muscles, with the levator ani at the lateral margins.

d. A photomicrograph of *b* and *c* demonstrating the mucosa of the vagina with its many folds, the tunica propria, the inner circular muscle, and the outer longitudinal muscle. In addition, the urethrovaginal septum and rectovaginal septum are shown.



PLATE I.

uterus in the region of the isthmus,^{15, 19, 26, 41} whereas a lesser number of fibers descends along the lateral aspects of the vagina. This pattern is similar to that of the arteries that supply the vagina. In addition to sending rami to the vagina, the vesical branches, in part, go directly to the bladder together with the blood supply.

The supports of the vagina have been discussed at length by many anatomists and surgeons. Because of their position as the connecting organ between the external genitalia and the uterus, its supports will have an intimate relationship with the urethra, bladder, and rectum. On gross dissection, the presence of large bands of tissue that radiate out to the lateral pelvic walls from the region of the isthmus of the cervix at the junction of the vagina with the uterus may be seen. These bands of tissue have been termed variously ligaments of Mackenrodt^{50, 57} and cardinal ligaments; posteriorly they are called sacrouterine ligaments and base of the broad ligament. They originate in the region of the junction of the isthmus of the uterine cervix with the corpus of the uterus^{50, 57, 74, 79} and course outward in a fanlike pattern to the lateral pelvic wall. The fibers of the isthmus appear to turn upward onto the uterus, as well as downward onto the vagina. The ligaments of Mackenrodt, the connective tissue surrounding the vessels on the lateral vaginal walls and the close proximity of the rectum, bladder, the urethrovaginal unit (with the puboprostatic ligaments) all contribute to support the vagina within the pelvis.

Microscopic Anatomy

The microscopic anatomy of the mucosa of the vagina is unique in its structure and in its response to the various hormones. The epithelium is of a thick (0.15 to 0.2 mm.)²⁹ uncornified stratified squamous type lacking any glands.^{33, 47, 51, 54, 63, 69} The transition of the squamous epithelium into the simple columnar epithelium of the endocervix is abrupt. The epithelium of the vagina consists of three main layers: a basilar layer composed of oval- to round-shaped cells with prominent nuclei; an intermediate layer of larger, flatter cells with nuclei; an inconstant zone of cornification; and a superficial layer of cornified cells with pyknotic nuclei that demonstrate response to hormonal stimulation.⁶⁶

The cyclic response of the epithelium to estrogen stimulation was first reported in the guinea pig by Stockard and Papanicolaou⁷³ and in the human by Dierks²¹. Since that time much work that further demonstrates the cyclic and hormonal variations in the vaginal epithelium has been done.^{1, 3, 44, 62, 72, 76} Electron microscopy of the vaginal epithelium is still a new method of study. Bahr and Moberger⁴ were able to show that the epithelial cells are connected syncytially with each other through bridges of protoplasm. In these areas no definite cell limits were observed. Bizzozero's nodules were observed in the protoplasmic bridges. Spaces under the protoplasmic bridges connected with each other to terminate finally in the lumen of the vagina. Densifications were seen in the epithelial cells; however, no keratinization was noticeable. The cytoplasm of the epithelial cells contained two types of fibrils, the coarser of which (tonofibrils) run from cell to cell, thus forming an integrating system of the epithelium.⁴

The lamina propria is relatively thicker than usually is seen (FIGURE 1).³³

It contains a thick network of collagenous fibers with an interlacing network of elastic fibers. Papillae indent the epithelium throughout its length, but more prominently on the posterior wall.^{33, 47, 51, 63, 66, 69} They are more prominent in the nulliparous individual and may play a significant role in aiding the epithelium to stretch during parturition. A submucosa which is loose in structure and somewhat indefinite is present.^{7, 14, 29, 33, 45, 47, 49, 63} Through this layer course the larger blood vessels and lymphatics, as well as the few nerves that supply the epithelium. The lymphatics may form small aggregates (intercalated nodes) similar to the Peyer's patches of the bowel.⁴² These aggregates may be observed also in the adventitia surrounding the organ, where occasionally they may be recognized on gross examination. The arteries in this area

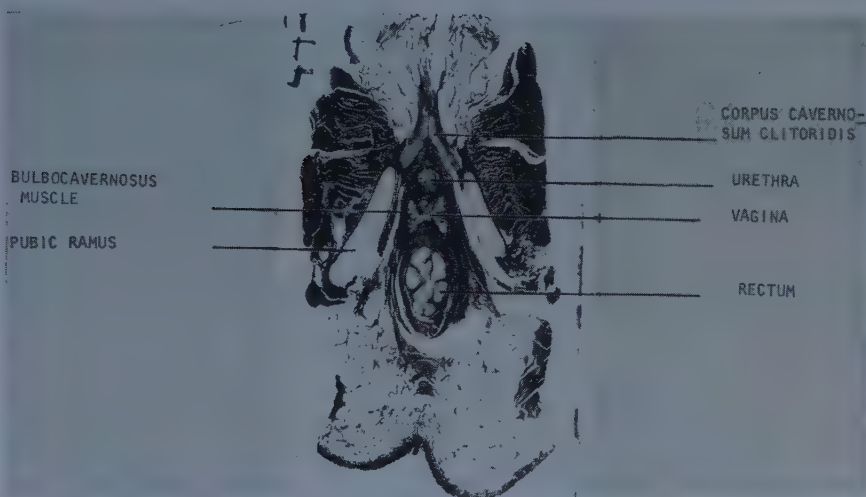


FIGURE 1. A section through the introitus demonstrating the urethra with its glands the vagina, and its many folds. On either side of the vagina is the bulbocavernosus muscle. The close relationship of the urethra and vagina, as well as of the vagina and the lower portion of the rectum, is apparent.

are tortuous in their course and appear somewhat spiral in nature, whereas the veins are of a more sinusoidal pattern. The looseness of the submucosa and its somewhat different vascular pattern may be a natural developmental pattern, similar to that observed in the epithelial rugae, to allow relatively sudden extreme distention during parturition.

The muscle pattern of the vagina has elicited considerable investigation. From the view of Henle,³³ who originally stated that the inner layer was longitudinal and the outer circular, to the opposite view held by Luschka,⁴⁷ many arguments have occurred (FIGURE 2).^{7, 9, 14, 29, 45, 47, 49, 63, 68} The muscle of the vagina is continuous with the muscle of the uterus.^{9, 63, 68} The outer layer of muscle of the uterus runs in a longitudinal fashion^{9, 68} and at the region of the isthmus (FIGURE 3), passes outward into the base of the broad ligament onto the lateral pelvic wall to form the superior surface of the cardinal ligaments. The outer layer of muscle of the vagina, longitudinal in direction,

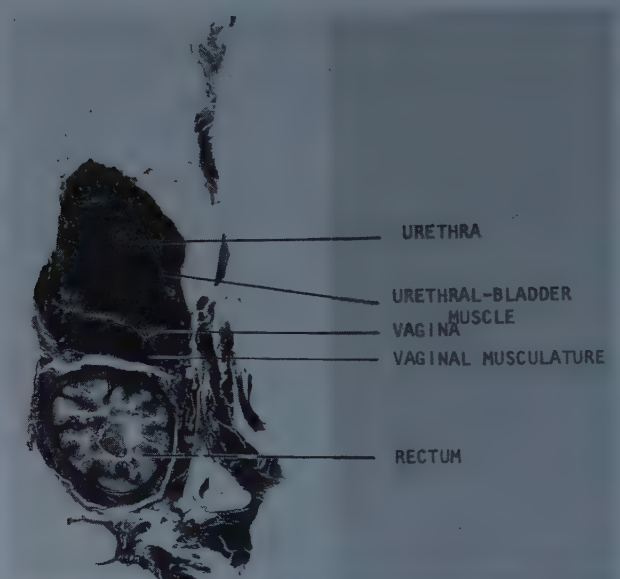


FIGURE 2. Section through the urethra, vagina, and rectum, demonstrating the close relationship of the urethra to the anterior vaginal wall. The section of urethra is from the junction of the middle and upper third.

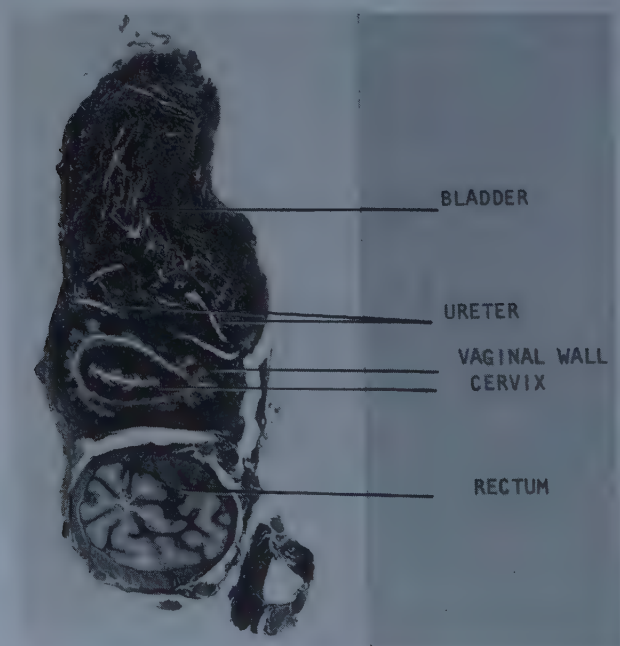


FIGURE 3. The relationship of the vagina, cervix, ureters, bladder, and rectum are demonstrated.

forms a similar pattern composing the inferior surface of the cardinal ligament. The muscle-to-connective-tissue ratio varies, although usually it is 1:3. The longitudinal muscle fibers continue along the length of the vagina to the region of the hymenal ring, where they gradually disappear in the connective tissue

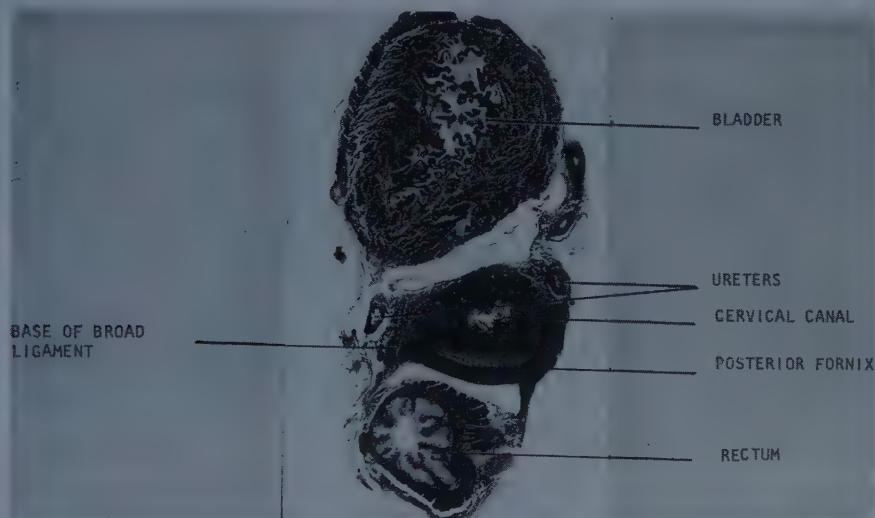


FIGURE 4. A section through the bladder, vaginocervical junction, and the base of the broad ligament demonstrating the relationship to the ureter, vagina, and cervix.

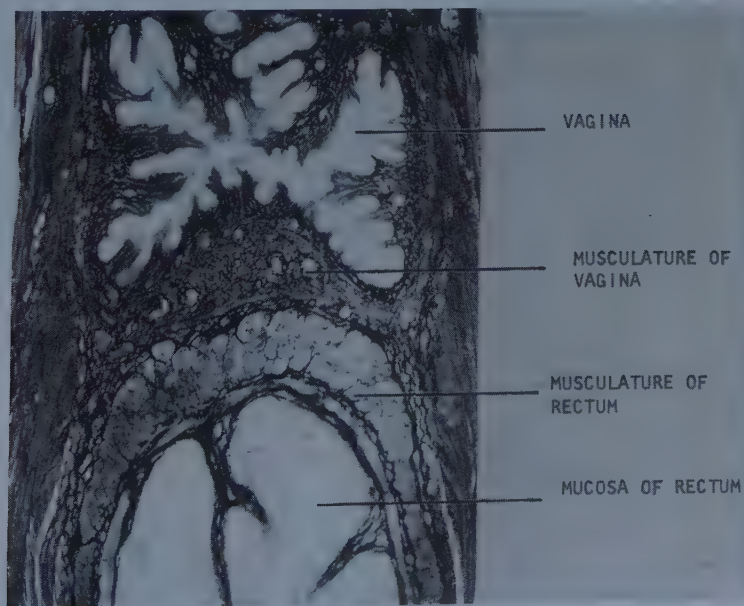


FIGURE 5. Section demonstrating the relationship between the vagina and rectum in the region of the hymenal ring.

of the area. On the anterior vaginal wall the longitudinal muscle fibers are found to be more displaced by the urethra than diminished in number (FIGURE 4).⁴¹ The inner muscle layer of the vagina is developed more poorly,^{29, 37, 41, 63, 68} forming a spiral-like course appearing in microscopic sections as somewhat circular in direction.^{41, 66, 68} In the region where the urethra traverses the anterior vaginal wall, the circular (spiral) fibers are less developed than are the

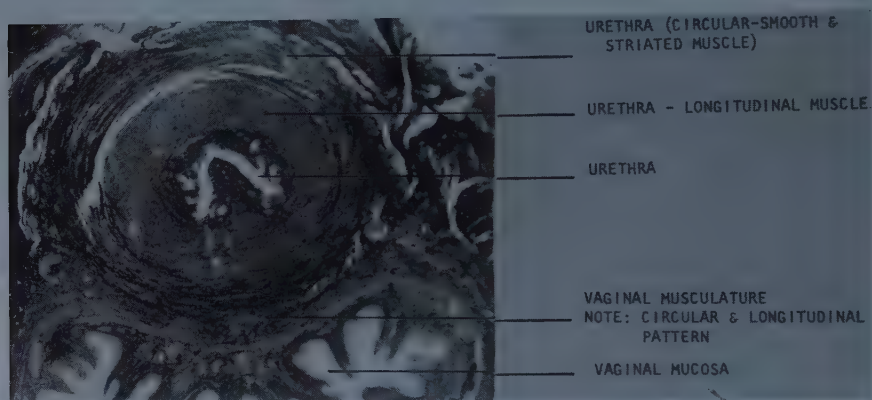


FIGURE 6. The anterior vaginal wall and urethra demonstrating the relationship between the two organs in the region of the striated sphincter of the urethra.

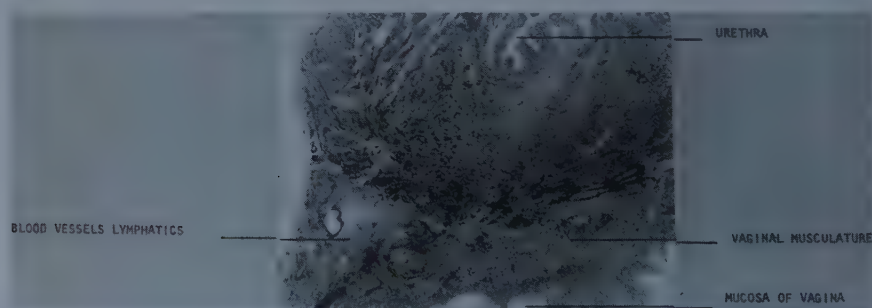


FIGURE 7. Section demonstrating the region of the junction of the upper third of the urethra and the anterior vaginal wall. The absence of a fascia, the separation of the two organs, the blood and lymphatic supply, and the lateral aspect of the junction of the two organs can be seen.

longitudinal. Striated fibers of the bulbocavernosus and ischiocavernosus muscles may be seen as they ascend from the lateral sides to surround the urethra and form the striated (voluntary) sphincter.⁴¹ These should not be confused with the vaginal circular musculature beneath the longitudinal smooth muscle of the vagina. The presence of fascia⁷⁰ surrounding the vagina and the presence of pubocervical fascia⁶ have been much disputed since first put forth by Bonney⁶ as a musculofascial plane. Although there are investigators who believe that such fascia exist,⁷⁷ there are others who dispute it.^{29, 37, 41, 63}

From my studies, both gross and microscopic, I believe that the adventitia of the vagina is composed of a loose areolar connective tissue. The presence of this connective tissue can be demonstrated on a cadaver; in living tissue it has little strength and contains mostly the blood vessels, nerves, and lymphatics to the vagina. I am dubious of the ability of the adventitia to play any role in the support of the bladder or rectum, which contain their own adventitia.

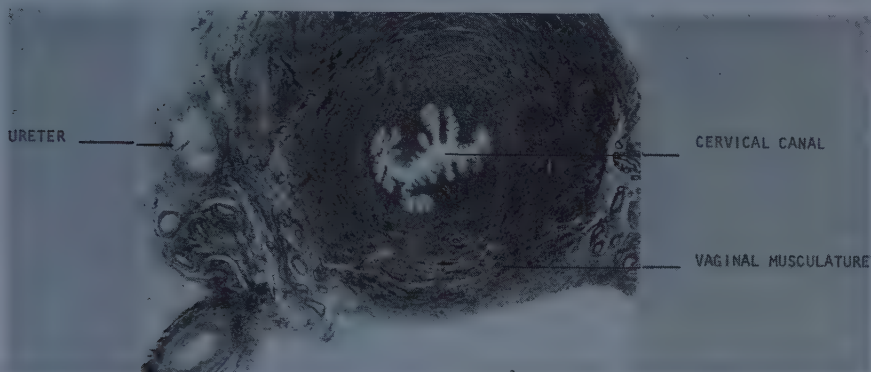


FIGURE 8. A section through the region of the junction of the vagina and isthmus of the cervix and the base of the broad ligament. Note the pattern of musculature.

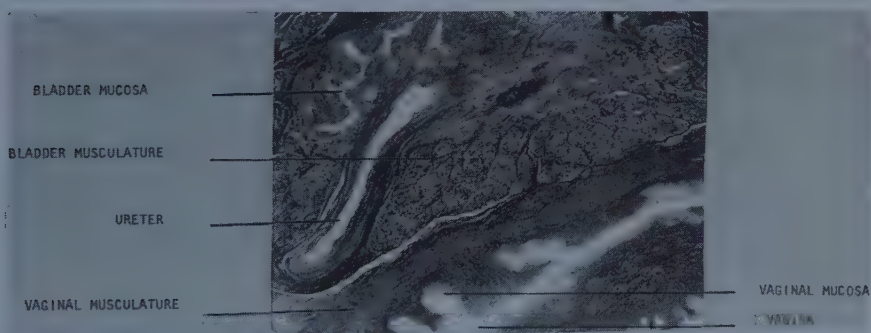


FIGURE 9. A section demonstrating the relationship of the ureter as it enters the bladder to the anterior vaginal wall. Note the apparent absence of any investing fascia between the bladder musculature and vaginal musculature.

Riehm⁶⁴ described definite intracellular edematous changes in the connective tissue of the vagina with a loss of fiber pattern during the process of parturition (FIGURES 5 to 11).

The microscopic study of the innervation of the vagina has not yielded many findings. Many investigators describe the presence of Vater-Pacinian-type corpuscles in the lamina propria of the vagina,^{2, 17, 22, 28, 40, 42, 48} although others have shown the apparent absence of these corpuscles.^{13, 25, 41} The presence of fine nerve endings to the blood vessels in the lamina propria has been

reported.^{59, 80} Connections between the ganglia seen in the adventitia of the vagina and the muscularis were reported by many early observers;^{17, 31, 48} one investigator stated that some of the fine fibers penetrate to the mucosa to terminate as free nerve endings (FIGURES 12 and 13). No demonstrable relationship between the nerve fibers in the vagina and responses of the mucosa to hormonal stimulation has been made.^{17, 34, 48} In my extensive studies⁴¹ occasional Vater-Pacinian-type corpuscles were observed in the adventitia surrounding the vagina, but none was found within the organ itself. Ganglia

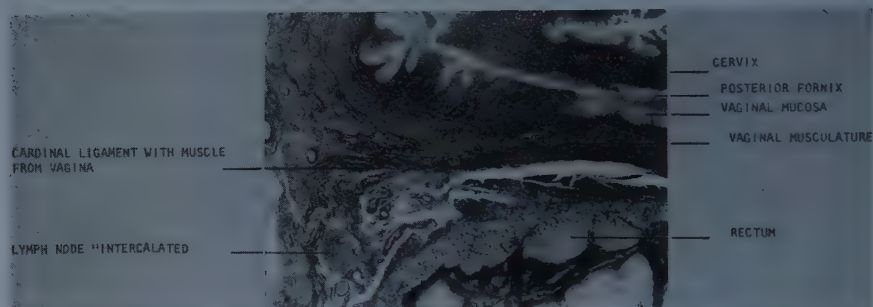


FIGURE 10. A section through the vagina, cervix, and rectum. The pattern of musculature of the vagina, as well as that of the rectum, is easily demonstrable. The absence of a fascia is apparent. The spread of vaginal musculature into the base of the broad ligament (cardinal ligament) is demonstrable.

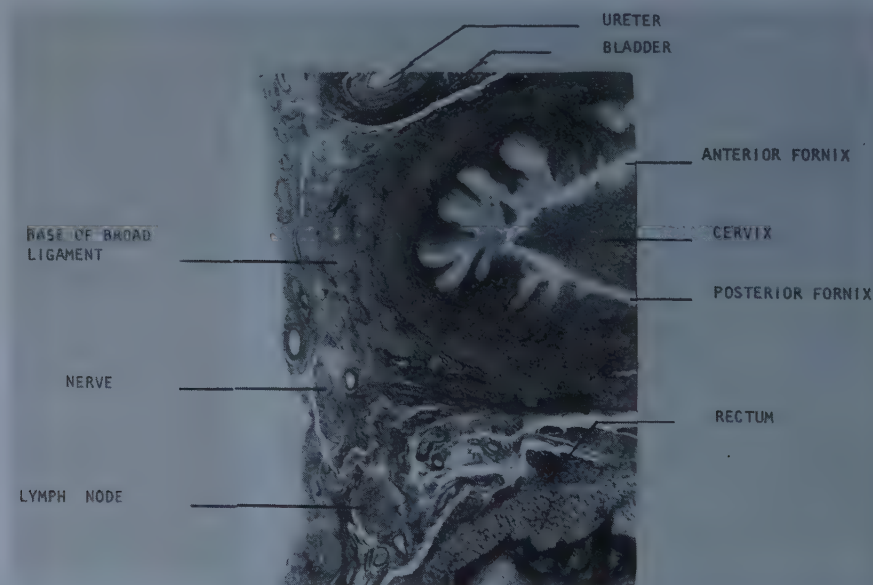


FIGURE 11. Section demonstrating the musculature of the vagina entering the broad ligament.

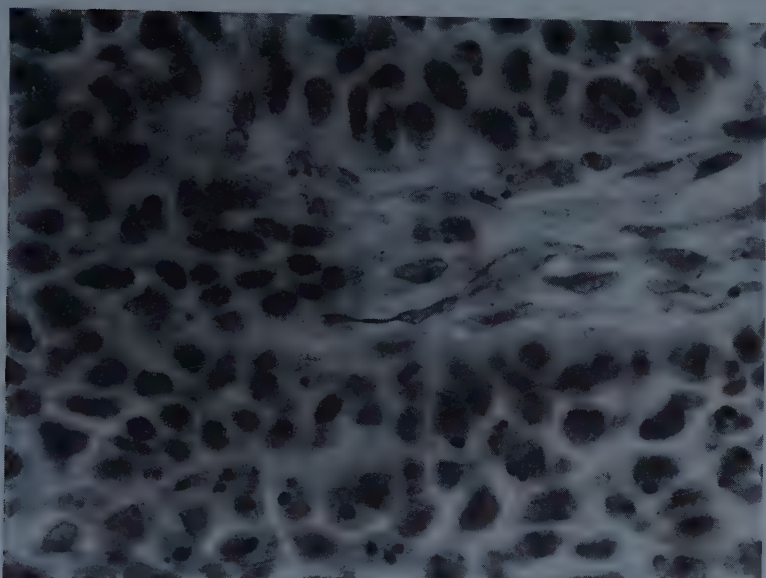


FIGURE 12. An arborization of a nerve ending in the vagina near the hymenal ring. Note the delicate structure as it divides into several elements. These are found with difficulty, due to the small number. Under the stratum germinativum, many of the observed endings terminate in the walls of the blood vessels. $\times 950$.

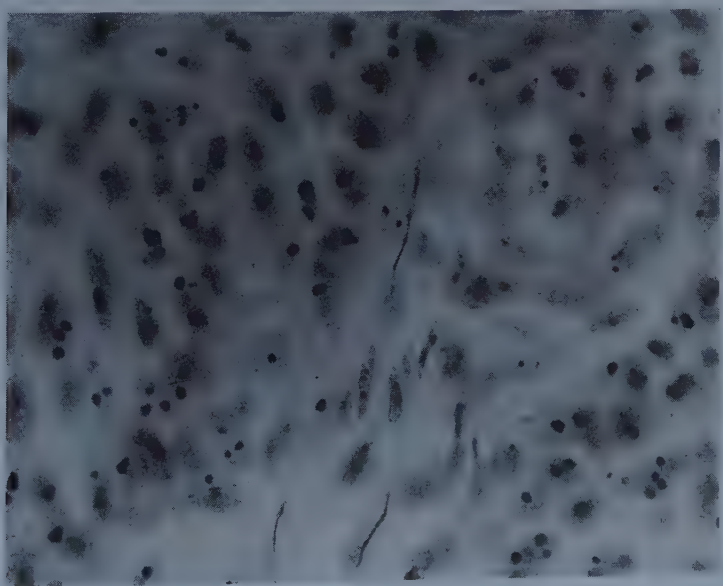


FIGURE 13. Terminal free nerve endings among the epithelial cells of the vaginal epithelium in the upper third of the vagina. When observed, they are usually in groups located in isolated areas with the greater number of the fibers remaining in the lamina propria. $\times 950$.

were found in the adventitia surrounding the vagina and situated along the lateral walls adjacent to the blood vessels. Fibers were noted to extend out to the ganglia of inferior hypogastric plexus. In the upper third of the vagina the ganglia frequently were situated between the bladder and vagina. The ganglia cells were both pseudouni- and multipolar. Unmyelinated fibers from, and passing through, the ganglia were observed to supply the muscularis and blood vessels; an occasional fiber penetrated to the mucosa and terminated in free endings in the basal layer. No other type of ending was observed. The nerves along and in the vagina revealed a wavy pattern similar to that observed by other investigators. This wave effect has been thought to be a protective device during the distention of the vagina in parturition.

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STUDIES ON VAGINAL ENZYMOLOGY*

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The enzyme β -glucuronidase has held our attention for many years.^{1a, b} *In vitro*, this enzyme catalyzes two reactions: one, the hydrolysis of glucuronic-acid conjugates,² and the other, the transfer of the glucuronyl radical from a donor glucosiduronic acid to an acceptor hydroxylated compound.^{3a, b} The hydrolysis of phenolphthalein glucosiduronic acid is employed for purposes of enzyme assay, the liberated phenolphthalein being measured readily in alkaline solution. The enzyme occurs widely in animal tissues, for example, liver, spleen, and the endocrine tissues, which are especially rich sources.

β -Glucuronidase and Gonadal Hormones

The following correlations have been found between β -glucuronidase and the action of gonadal hormones. Upon ovariectomy, the uterine β -glucuronidase diminishes to one half the value of that in intact control mice and rats. After estrogen administration, the enzyme level is restored to normal.⁴ These findings have been confirmed and extended by other investigators.⁵ Thus, Knobil, in 1952,⁶ found that rat-mammary-gland β -glucuronidase and Beyler and Szego⁷ observed that rat preputial gland, upon castration and subsequent estrogen administration exhibited behavior similar to that of the uterus. In the human, uterine endometrium is rich in β -glucuronidase, its concentration being especially high in the last part of the menstrual cycle.⁸ In pregnancy, serum β -glucuronidase becomes elevated and returns to normal after delivery.^{9, 10} Stilbestrol can prevent the postpartum fall in β -glucuronidase.¹¹ In postmenopausal women, the serum β -glucuronidase responds to small amounts of estrogen.¹² The preceding facts amply demonstrate a correlation of this enzyme with the action of estrogenic hormones.

With regard to androgenic hormones, it was discovered in 1951 that testosterone caused a marked increase in the β -glucuronidase activity of mouse kidney, which is a target organ for androgen.¹³ The effect could be demonstrated readily by the administration of luteinizing hormone to intact male mice. Moreover, in subjects receiving androgens¹⁴ elevations in serum β -glucuronidase have been observed; detailed studies have appeared.¹⁵⁻¹⁷

Vaginal Fluid β -Glucuronidase Activity

The correlation of data on high β -glucuronidase activity in many tumors led us to the study of vaginal fluid in the following manner. Based on the β -glucuronidase-rich cancers of the cervix, Odell *et al.*^{18, 19} postulated that the enzyme would leak out to enrich the vaginal fluid. They proposed that measurements of vaginal-fluid β -glucuronidase activity could be used as a

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diagnostic aid in detecting cervical cancer. In the course of studies undertaken to evaluate the claims of Odell and his associates, it was learned that the vaginal fluid β -glucuronidase was under the control of a number of physiological and pathological factors.

Thus, with regard to ovarian function it was observed that there is a significantly greater incidence of high β -glucuronidase in the vaginal fluid in women after the menopause;²⁰ that there is a cyclic variation of vaginal-fluid β -glucuronidase related to the events of the menstrual cycle; that following ovariectomy or massive X-ray irradiation of the ovaries, elevated enzyme values result; and that the administration of estrogen depresses vaginal fluid β -glucuronidase.²¹

The major source of this enzyme in the vaginal fluid would appear to be the vaginal mucosa itself. This statement is based on the fact that panhysterectomized women, lacking cervix and uterus, frequently show high β -glucuronidase titers in the vaginal fluid.

However, in the individual whose reproductive system is intact, the source of the vaginal fluid enzyme may be one or more of the following: (1) vaginal mucosa, (2) uterine cervix, (3) uterine endometrium, (4) bacterial flora, (5) blood, lymph, and tissue fluids, (6) cancer, (7) protozoa, (8) benign erosions and inflammations, (9) urine reflux, (10) extrauterine organs (ovary and fallopian tubes), and (11) gestational tissues.

Vaginal Fluid Enzymology

This array of sources of enzyme suggests the possibility that some indication of the state of these various tissues might be forthcoming from a knowledge of the activity of certain enzymes in the vaginal fluid, a concept very much in harmony with the one of measuring enzymes in the blood as an index of the activity of individual organs.²² This approach is one we are anxious to develop in relation to reproductive physiology.

It seemed to us that a first prerequisite was to have some knowledge of the tissue location of a number of enzymes in the reproductive tract. For this objective, the techniques of enzyme histochemistry are essential.

Enzymomorphology

Our objectives have been (1) to devise a uniform technique of fixation so that the integrity of tissue enzymes and of cell morphology is preserved, (2) to determine the time limit within which it is possible to process the tissue, (3) to be in a position to repeat observations on a single tissue specimen, and (4) to make serial sections which would permit the evaluation of the enzyme picture in a minute specimen of tissue.

The secret is the use of formaldehyde fixative solution to which was added a small amount of chloral hydrate^{23, 24} and the use of gelatin embedding of the fixed tissue 24 hours later.²⁵ The gelatin-tissue block is mounted on the freezing microtome and sections as thin as 5 μ may be cut with the knife. These sections are collected in 5 containers, 10 sections to each. From each vessel one section is taken to be stained with hematoxylin and eosin, and from a survey of these representative sections a selection is made of the compartment of sections considered most interesting for investigation. Two sections are

employed for the localization of each enzyme, one of them being counter-stained later with a suitable counterstain to aid in the interpretation. The enzymes studied and methods for localization were as follows: α -naphthyl esterase, Nachlas and Seligman;²⁶ acid phosphatase, Rutenberg and Seligman;²⁷ alkaline phosphatase, Kaplow;²⁸ β -glucuronidase, Fishman and Baker's²³ version* of the method of Friedenwald and Becker,²⁹ and reduced diphosphopyridine nucleotide diaphorase (DPNH-diaphorase), Farber *et al.*³⁰

We decided to make a systematic survey of the reproductive tissues of the human female by first examining specimens of vagina, cervix, and uterus removed surgically for benign pathology, for example fibroids. We have completed the study of more than 15 such specimens, but are reporting on our results in only 7 women who still were having menstrual periods and in 3 women

TABLE 1
VAGINAL ENZYMORPHOLOGY

Patient	Age	Day of menstrual period	Condition
Premenopausal subjects			
B.S.	37	3	Leiomyomata uteri
E.H.	44	10	Adenomyosis uteri
R.G.	48	11	Leiomyomata uteri
H.L.	35	12	Diverticulitis of large bowel
M.B.	47	13	Leiomyomata uteri
L.R.	39	18	Leiomyomata uteri
I.L.	48	23	Multiple leiomyomata
		Years postmenopause	
Postmenopausal subjects			
U.S.	51	5	Uterine prolapse (vaginal hysterectomy)
M.P.	67	20	Pseudomucinous cystadenoma
A.B.	71	25	Uterine prolapse

who were at least five years postmenopausal. Also, we are presenting only the results on vaginal tissues.

Vaginal Enzymorphology

TABLE 1 lists the subjects in order of days from day 1 of the menstrual cycle and years after the menopause, as the case may apply; each case is illustrated in FIGURES 1 to 10. Finally, observations on the vagina of a case of Stein-Leventhal syndrome are presented in FIGURES 11 to 13.

The tissue sites of each enzyme studied are characteristic. Thus, esterase activity is found consistently in the superficial and basal layers of the vaginal epithelium (FIGURES 1a, 2a, 3a, 4a, 5a, 6a, 7a, 8a, 9a, 10a); in later stages of the menstrual period and in postmenopausal women, the upper intermediate layers of the epithelium show intense activity. (FIGURES 4a, 5a, 6a, 6c, 7a, 8a, 10a). The lamina propria and connective tissue stroma are usually devoid of esterase activity.

* The substrate, 8-hydroxyquinoline glucosiduronic acid, is available from the California Corporation for Biochemical Research, Los Angeles, Calif.

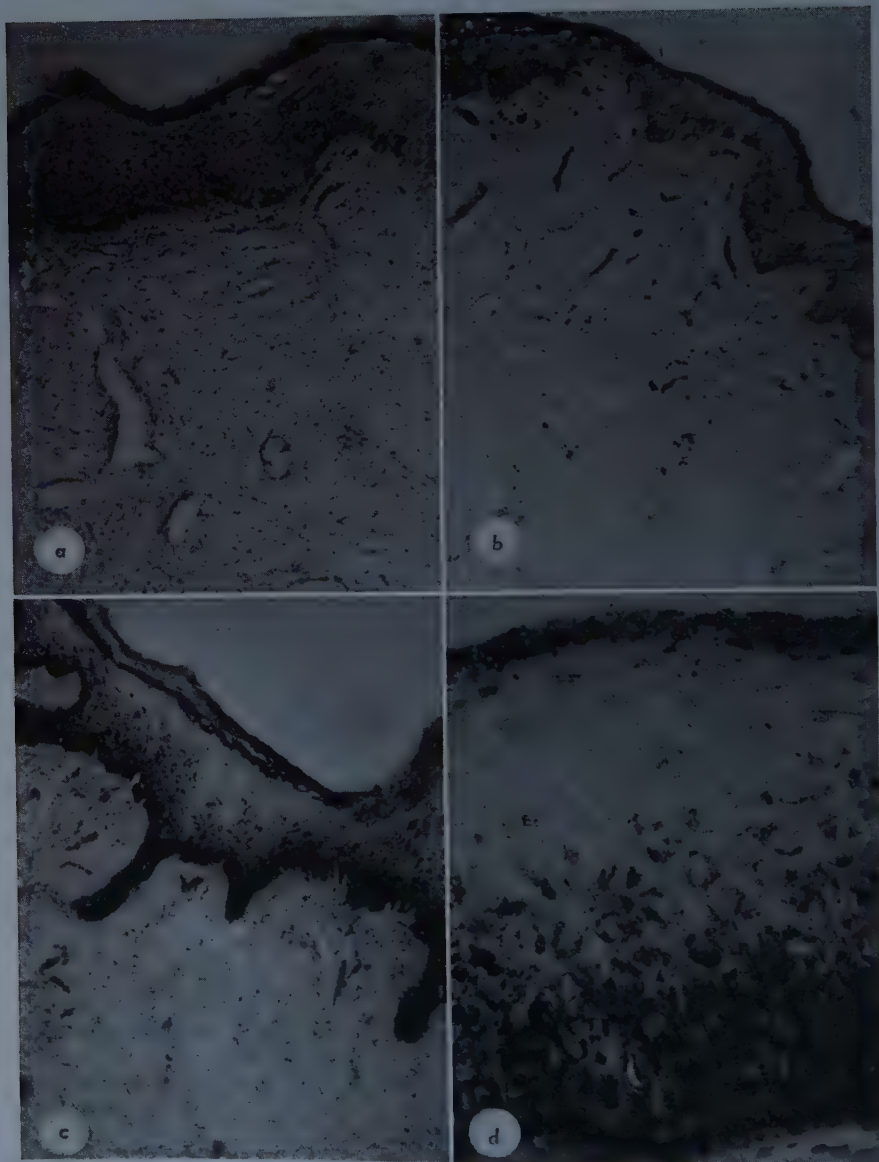


FIGURE 1. (a) Esterase (counterstained with Feulgen). Case B.S. (b) Acid phosphatase. Note activity in stromal erythrocytes. Case B.S. (c) β -Glucuronidase. Very intense activity in the cytoplasm of cells of the basal layers as well as in the superficial zone; capillaries positive. Feulgen counterstain. Case B.S. (d) β -Glucuronidase. Case B.S. Higher magnification ($\times 400$) of FIGURE 1c.

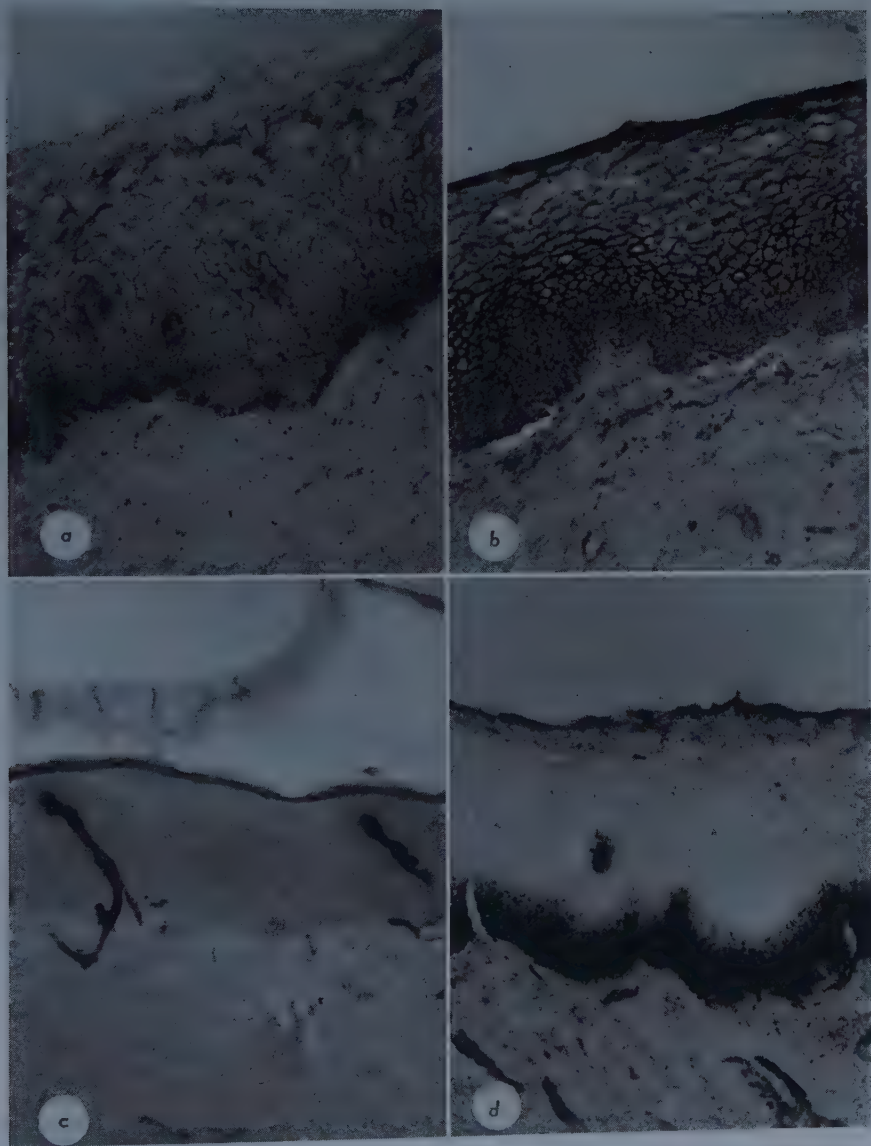


FIGURE 2. (a) Esterase. Basal layer and superficial layers positive; except for macrophages and some fibroblasts, the lamina propria is negative. Case E.H. (b) Acid phosphatase. Particularly strong reaction is evident in the superficial and middle intermediate layers of the mucosa. Note positive nuclei; little activity in the lamina propria. Case E.H. (c) Alkaline phosphatase. Capillaries are strongly positive (the superficial layer of the mucosa photographs black, but in reality is dark-yellow and therefore negative). Case E.H. (d) β -Glucuronidase. Basal and lower intermediate layers of the mucosa exhibit a strong reaction, as do the stromal capillaries. The superficial layer is comparatively enzyme poor. Case E.H.



FIGURE 3. (a) Esterase. Strongly positive superficial layer. Case R.G. (b) Acid phosphatase. Vaginal mucosa strongly positive, except for weak activity in the basal layer. Stroma negative, except for erythrocytes. Case R.G. (c) Alkaline phosphatase. Only capillaries are positive. Case R.G. (d) DPNH-diaphorase. Both mucosa and lamina propria show a moderate reaction. Case R.G. (e) DPNH-diaphorase. Stroma elements (capillaries, macrophages) are positive. Case R.G. (f) β -Glucuronidase. Basal and lower intermediate layers are strongly positive. In the latter, unusually large cells are present. The superficial layer is comparatively weak in reaction. Case R.G.



FIGURE 4. (a) Esterase. Marked reaction is more evident in the superficial layer and in portions of the upper-intermediate layers than in the rest of the mucosa. Case H.L. (b) Acid phosphatase. Intense enzyme activity in the mucosa, especially in the superficial layer. Red blood cells strongly positive; stromal elements weakly positive. Case H.L. (c) Alkaline phosphatase. Capillaries are strongly positive; everything else, including the superficial layer of the mucosa, registers negative. Case H.L. (d) DPNH-diaphorase. Both mucosa and lamina propria are reactive, the mucosa much more intensely. The upper intermediate layer is somewhat poorer in enzyme than the other layers; the capillaries are outstanding. Case H.L. (e) β -Glucuronidase. Superficial layers of the mucosa are moderately reactive; upper and middle-intermediate layers, negative; lower-intermediate and basal layers, very strongly positive. Lamina propria are weakly positive, except for the capillaries, which show a reaction of medium intensity. Case H.L.



FIGURE 5. (a) Esterase. Intense reaction in the mucosa, but not as intense as the yellow pigment (negative for esterase) in the section which photographs black. More enzyme activity appears in the superficial than in the basal layer, and more in the basal than in the other layers. Most of the lamina propria is negative. Case M.B. (b) Acid phosphatase. Mucosa and blood cells are strongly positive; lamina propria, very weakly reactive. Case M.B. (c) Alkaline phosphatase. Capillaries are intensely positive; other elements of the lamina propria and the mucosa are negative. Case M.B. (d) DPNH-diaphorase. In the mucosa, the superficial and basal layers are stronger than the other layers. The upper-superficial layer is weakly positive. The lamina propria is intensely reactive, especially the capillaries. Case M.B. (e) β -Glucuronidase. Superficial, lower-intermediate, and basal layers are positive; upper and middle-intermediate layers are negative. In the lamina propria, only the blood capillaries and a few macrophages are positive. Case M.B.

The distribution of acid phosphatase shows a localization to the vaginal mucosa with the erythrocytes in blood capillaries exhibiting rich acid phosphatase activity in an enzyme-poor lamina propria (FIGURES 1*b*, 2*b*, 3*c*, 4*b*, 5*b*, 6*c*, 7*b*, 8*b*, 9*b*, 10*c*). Of the individual layers of cells in the mucosa, the basal layer is usually weakest in enzyme activity. Occasionally, enzyme-positive nuclei may be seen in the mucosa (FIGURE 2*b*). The most intense reactions have been observed in the latter half of the menstrual cycle (FIGURE 7*b*) and in postmenopausal women (FIGURES 8*b*, *c*).



FIGURE 6. (a) Esterase. Superficial layer of the mucosa is strongly positive; the other layers are weakly reactive. Macrophages in the lamina propria are enzyme-positive. Case L.R. (b) Acid phosphatase. Mucosa is moderately to strongly reactive. Red blood cells are strongly positive; very weak enzyme activity in the lamina propria. Case L.R. (c) β -Glucuronidase (hematoxylin counterstain). Superficial, lower-intermediate, and basal layers of the mucosa show intense enzyme activity. Scattered cells in the other layers are also strongly positive. The lamina propria is weakly reactive. A small blood vessel shows moderate activity. Case L.R.

The alkaline phosphatase is localized by the method of Kaplow only in the endothelial cells of the capillaries nourishing the vaginal mucosa (FIGURES 2*c*, 3*c*, 4*c*, 5*c*, 7*c*, 8*c*, 9*c*).

In the case of β -glucuronidase, evidence of strong activity is found always in the basal germinal layer and often in the several layers of cells above this one (FIGURES 1*c*, 2*d*, 3*f*, 4*e*, 5*e*, 6*c*, 7*d*, 8*e*, 9*e*, 10*c*). Variable activity is observed also in the superficial layer (FIGURES 1*d*, 3*f*, 6*c*). In the stroma, the endothelial cells of the capillaries always show intense activity (FIGURE 10*c*), as do stromal cells on occasion (FIGURE 8*e*). It appeared as if stromal β -glucuronidase was quite active in postmenopausal women.

DPNH-diaphorase is widely and evenly distributed among the tissue and

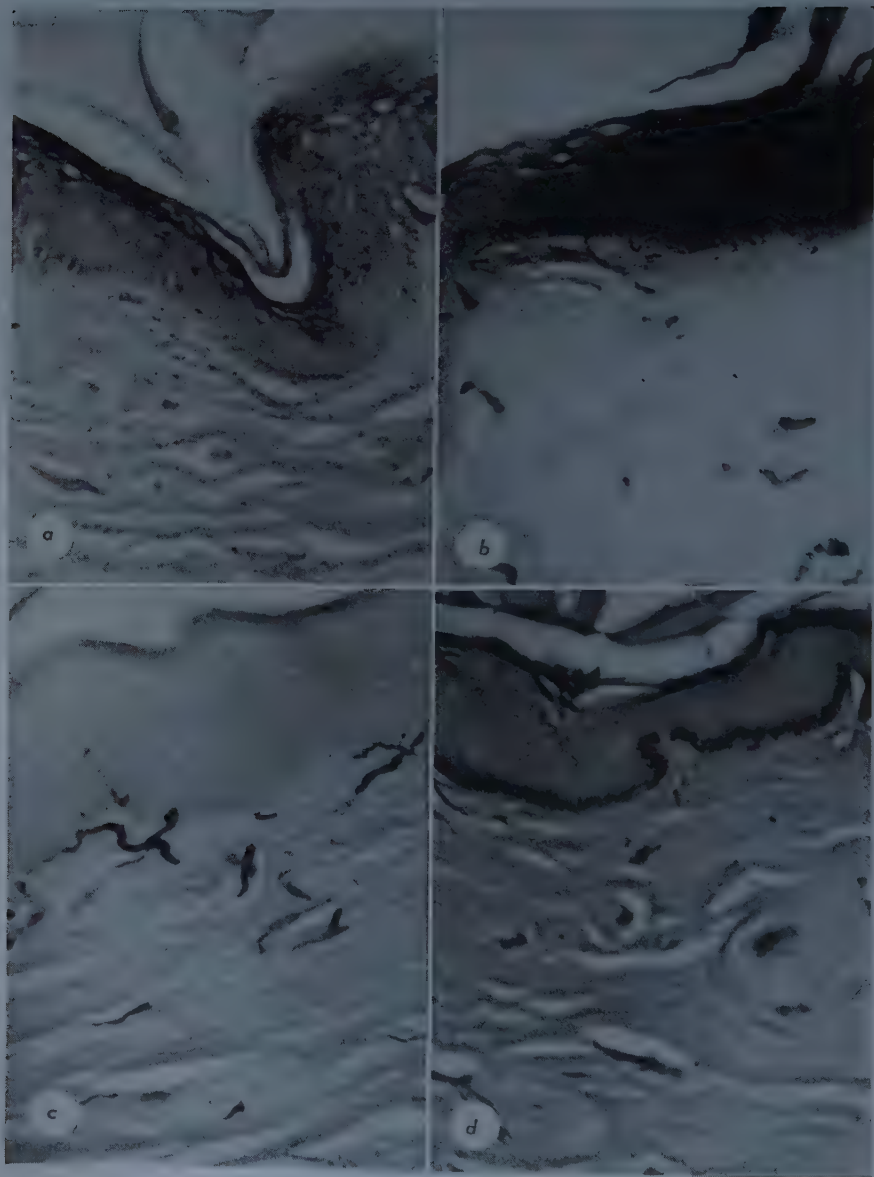


FIGURE 7. (a) Esterase. Superficial layer of mucosa is moderately reactive. The other layers are weakly positive; lamina propria is negative. Case I.L. (b) Acid phosphatase. Mucosa and red blood cells, strongly positive. Lamina propria shows weak enzyme activity. Case I.L. (c) Alkaline phosphatase. Capillaries are strongly positive; everything else is negative. Case I.L. (d) β -Glucuronidase. Superficial, basal layers of mucosa, and capillaries are strongly reactive; everything else is negative. Case I.L.



FIGURE 8. (a) Esterase. Superficial layer of mucosa is intensely reactive; other layers are weakly to moderately positive. Fibroblasts and macrophages in the lamina propria react strongly. Case U.S. (b) Acid phosphatase. Extremely intense reaction occurs in the mucosa and red blood cells. Lamina propria is weakly positive. Case U.S. (c) Alkaline phosphatase. Only the blood capillaries are positive (in the section, the superficial layer of the mucosa is dark yellow, not black). Case U.S. (d) DPNH-diaphorase. Moderate enzyme activity is present in the superficial and basal layers of the mucosa. Weak reaction is noted in the other layers. The capillaries are outstanding in the otherwise weakly positive lamina propria. Case U.S. (e) β -Glucuronidase. Enzyme activity is stronger in the basal layer than in the other layers of the mucosa. The intermediate layer is weakly positive. The lamina propria is intensely reactive. Case U.S.

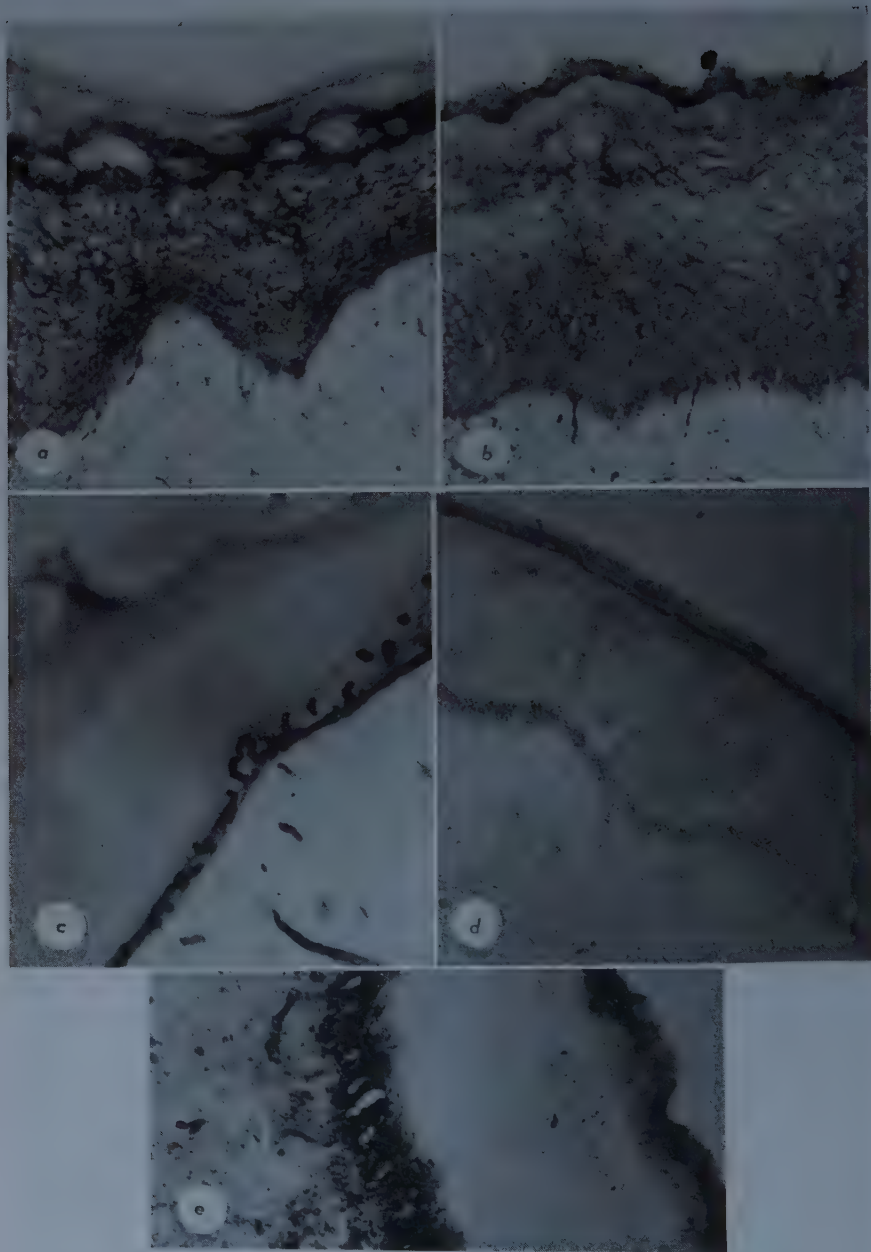


FIGURE 9. (a) Esterase. More intense reaction is observed in the upper-intermediate than in the other layers of the mucosa. The superficial layer is negative. A few cells in the lamina propria are positive. Case M.P. (b) Acid phosphatase. Mucosa is moderately positive. Weak enzyme activity is found in the middle-intermediate layer. Reaction in the red blood cells varies from moderate to strong. The lamina propria reacts very faintly. Case M.P. (c) Alkaline phosphatase. Capillaries only are strongly positive. Case M.P. (d) DPNH-diaphorase. Moderate enzyme activity is present both in the mucosa and in the lamina propria. In the mucosa the reaction is strongest in the superficial layer, and further, is more intense in the basal than in the intermediate layer. Case M.P. (e) β -Glucuronidase. Very intense reaction is noted in the basal and lower-intermediate layers, and a less strong reaction in the superficial layer; the other layers are practically inactive. Capillaries and some stromal cells are rich in enzyme. Case M.P.

cellular elements of the vagina, occurring in the basal, intermediate, and superficial layers of the mucosa, and in capillaries and stromal cells of the lamina propria (FIGURES 4*d* and 5*d*). In the two postmenopausal subjects studied (FIGURES 8*d* and 9*d*), this activity seemed to be depressed uniformly. These results would appear to fit the findings of Rosa and Velardo on DPNH-diaphorase of rodent vagina in the absence and presence of estrogen.

A small specimen of vagina was obtained from a 23-year-old patient exhibiting Stein-Leventhal syndrome, which is manifested by prolonged anovulation.

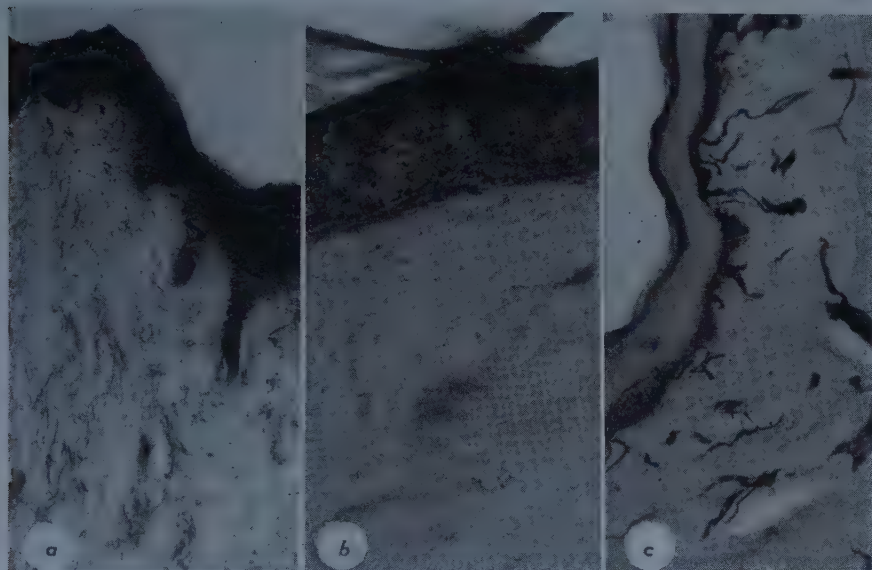


FIGURE 10. (a) Esterase. Superficial, upper, and middle-intermediate layers of the mucosa are strongly positive. The other layers are weakly reactive (black and white photographs give a false impression of the actual intensity of the esterase localization). Lamina propria is negative. Case A.B. (b) Acid phosphatase. Strong enzyme activity is present in all layers of the mucosa, especially in the superficial layer, the stroma being weakly positive. Red blood cells are moderately reactive. Case A.B. (c) β -Glucuronidase. Superficial and basal layers of the mucosa are strongly positive, making a sharp contrast with the other layers, which are completely unreactive. Capillaries show intense enzyme activity; the rest of the lamina propria is negative. Case A.B.

The findings for β -glucuronidase, alkaline phosphatase, DPNH-diaphorase, and acid phosphatase are illustrated in FIGURES 12*b, c, d* and 13*a, b, c*.

It would appear that each of the tissue sites of activity of the five enzymes investigated has a characteristic location; there are indications that, except for alkaline phosphatase, it may be possible in the future to draw correlations with the state of ovarian function.

Summary

It is proposed that, just as a knowledge of the levels of certain blood enzymes is important to the internist, so perhaps the level of vaginal fluid enzymes may provide new objective data for the gynecologist. The source of the vaginal

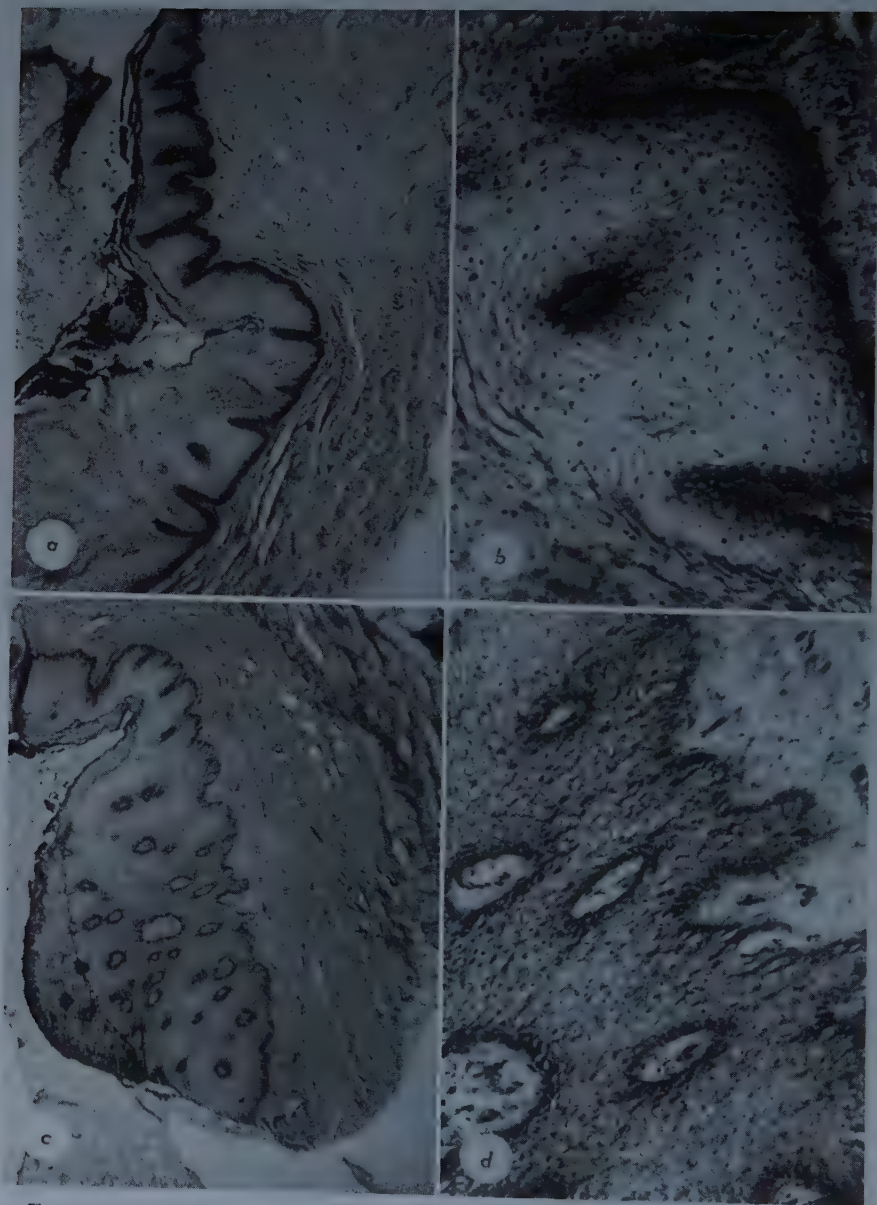


FIGURE 11. (a) β -Glucuronidase. The enzyme activity is limited to the superficial and basal layers. Case C.S. (b) β -Glucuronidase (hematoxylin counterstain); section corresponding to *a* is shown at high magnification; strong reaction in the basal layer. Case C.S. (c) β -Glucuronidase. Note moderate reaction in the mucosa; the basal layer reacts more intensely than the other layers. Case C.S. (d) β -Glucuronidase. Shown is a high magnification of specimen in *c*. Case C.S.

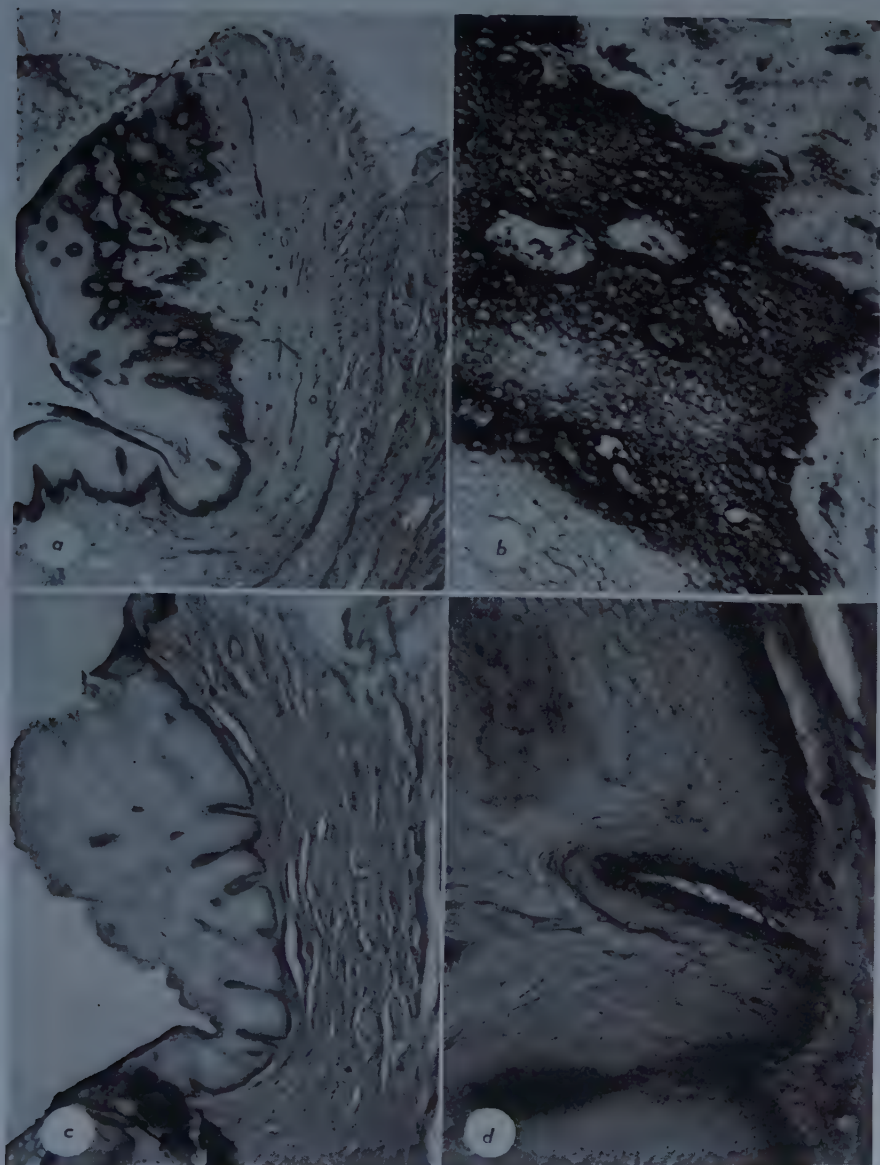


FIGURE 12. (a) β -Glucuronidase. Intense enzyme activity is noted in the basal layer of the mucosa. In the area showing discrete hyperplasia, a strong reaction is also exhibited by the intermediate layer. Blood capillaries are also positive. Case C.S. (b) β Glucuronidase. Shown is a high magnification of the specimen in (a). Case C.S. (c) β -Glucuronidase. Strong enzyme activity is seen in the basal layer of the mucosa; in most places the rest of the mucosa is negative. Case C.S. (d) β -Glucuronidase. Details of the specimen in c are shown. Case C.S.

fluid enzyme may be one or more of the following: vaginal mucosa, uterine cervix, uterine endometrium, bacterial flora, blood lymph and tissue fluids, cancer, protozoa, benign erosions and inflammations, urine reflux, extrauterine organs, and gestational tissues.

With regard to the vaginal fluid enzymes, a systematic study has been under way for a few years on the relationship of the enzyme β -glucuronidase to physiological (menstrual cycle, menopause, pregnancy) and to pathological (inflammatory disease, trichomonas, and cancer) states of the vagina and cervix. These observations have illustrated the difficulties of interpretation of the



FIGURE 13. (a) Alkaline phosphatase. Only the capillaries are positive. Case C.S. (b) Acid phosphatase. Enzyme concentration is more intense in the superficial, and in portions of the basal, layer than in the other layers of the mucosa. Red blood cells are strongly positive. Stromal elements show a weak reaction. Case C.S. (c) DPNH-diaphorase. Basal layer of the mucosa is strongly positive. Other layers and the lamina propria show weak to moderate enzyme activity. Case C.S.

significance of individual values in the absence of an exact knowledge of the source of the enzyme.

It is for the purpose of gathering information that may bear on this point that we have undertaken an intensive study of the enzyme histochemistry of the vagina, cervix, and endometrium.

Vaginal tissue sites for activity of β -glucuronidase, acid phosphatase, alkaline phosphatase, DPNH-diaphorase, and esterase have been visualized in 7 premenopausal women and in 3 postmenopausal women. For each enzyme, a characteristic tissue pattern is obtained in the various layers of the epithelium, in the capillaries, red and white cells, and in the connective tissue stroma. For example, in the case of β -glucuronidase, the basal germinal layer of the epithelium is richest; for esterase, the superficial epithelium; for acid phosphatase,

the middle and superficial layers of the epithelium and red cells; and for alkaline phosphatase, the endothelial cells of the capillaries. For DPNH-diaphorase, an even distribution of activity in the epithelium and stroma exists. The findings are illustrated in 54 microphotographs. Certain findings suggested that in the future it may be possible to draw correlations between enzyme sites and activity with ovarian function.

Acknowledgments

We thank Kathleen T. Ladue and Paulo Borges for their valuable assistance.

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HISTOCHEMICAL LOCALIZATION OF VAGINAL OXIDATIVE ENZYMES AND MUCINS IN RATS TREATED WITH ESTRADIOL AND PROGESTERONE*

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Introduction

Any treatment of the basic biology of the vagina eventually must define the physicochemical mechanisms that underlie the various levels of functions in this organ structure. Also, because of the demonstrated dependence of the vaginal structural and functional components upon a rigorously defined yet varying level of circulating steroid hormones, a consideration of the interplay of such hormones and their subsequent manifestations on these mechanisms is essential. Very recently biochemical endocrinology has elucidated some of the initial interactions between ovarian steroids and certain critical enzymes, the transdehydrogenases (Talalay and Williams-Ashman, 1958). The net effect of these studies has been to indicate the most probable means by which cells in target structures, such as the uterus and vagina, initiate enzymatic mechanisms that involve an increase in available high-energy phosphate esters. The presence of these organic phosphate compounds is imperative for the expression of the induced changes in these organs by the steroid hormones and their metabolites acting in concert (Velardo, 1958*a, b*; 1959). Enzymes such as the diphosphopyridine-nucleotide- and triphosphopyridine-nucleotide-linked dehydrogenases, succinic dehydrogenase, and their respective systems are part of a group that effects certain specific biological oxidation-reduction reactions, the most distinguishing feature of which is the transfer of parceled energy into the phosphate bonds of compounds such as adenosine triphosphate (ATP) and creatine phosphate.

The convergence of such disciplines as histochemistry and experimental endocrinology on problems related to the effects of hormones on target oxidative enzyme systems has resulted in the demonstration within the uterus and the vagina of "pattern distributions" that are characteristic for a given enzyme and for specific organ regions (Rosa and Velardo, 1958). These studies, made with the use of the light microscope, have permitted the identification of active and relatively inactive regions within the vaginal epithelium. In addition, other investigations have indicated that certain histochemically demonstrable oxidative enzymes in the uterus and the vagina of the rat are apparently under the control of circulating titers of ovarian hormones and their metabolites during the estrous cycle (Rosa and Velardo, 1959). It is the purpose of this presentation to present further experimental evidence to this effect. However,

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our plan of study involves the use of administered ovarian sex steroids to castrated animals. The influence of estradiol-17 β and progesterone used either singly or in combination upon the succinic dehydrogenase system (SDH), diphosphopyridine nucleotide-diaphorase system (DPN), and mucin formation in the vaginal epithelium has been assessed histochemically. Specifically, this investigation has been an attempt to study the divorced and combined effects of these sex steroids on the oxidative metabolism and mucinogenesis of the component layers of the vaginal epithelium of the rat. In addition, certain preliminary observations by us have implied an association between the presence of vaginal mucins (Velardo *et al.*, 1953b) and DPN-diaphorase (Rosa and Velardo, 1959); we shall present more evidence for our earlier comments.

Materials and Methods

Mature albino rats (Sprague-Dawley strain) weighing between 190 and 210 gm. were ovariectomized. These animals were allowed free access to water and laboratory chow (Purina) containing normal vitamin requirements. The treated animals received hormonal injections once daily for three days beginning with the seventh day after ovariectomy. All rats were sacrificed by concussion ten days after ovariectomy (that is, in the hormone-treated series, 24 hours after the last injection). Vaginas were instantly removed, frozen upon dry ice, and maintained in the frozen state until studied for oxidative enzymes and vaginal mucification. Only areas midway between the ectocervix and vaginal os were studied.

Estradiol-17 β and progesterone were the ovarian steroid hormones used in this study. Graded concentrations of each were prepared in sesame oil and injected subcutaneously in the interscapular region in 0.1-ml. quantities. The graded daily dosages of each hormone were as follows:

Estradiol-17 β : 0.005, 0.01, 0.025, 0.05, 0.10, 0.5, and 1.0 μ g.

Progesterone: 0.05, 0.10, 0.25, 0.50, 0.75, 1.0, 1.5, and 4.5 mg.

(The effects of the last two concentrations of progesterone were not studied in the mucification series.) In addition to the above, combinations of 0.1 μ g. estradiol-17 β and varying concentrations of progesterone were injected. In these cases, the two steroids were injected separately and into more lateral sites.

The 3-day daily injections of combined hormones were as follows:

0.10 μ g. estradiol-17 β plus 0.05 mg. progesterone

0.10 μ g. estradiol-17 β plus 0.25 mg. progesterone

0.10 μ g. estradiol-17 β plus 0.5 mg. progesterone

0.10 μ g. estradiol-17 β plus 1.0 mg. progesterone

0.10 μ g. estradiol-17 β plus 1.5 mg. progesterone

0.10 μ g. estradiol-17 β plus 4.5 mg. progesterone

Tissues were studied from groups of animals ranging from 8 to 12 in number following separate or combined treatments.

For the localization of the SDH system and the DPN systems, the histo-

chemical techniques employed were the same as those described previously (Rosa and Velardo, 1954, 1959). Briefly, these involve the use of certain tetrazolium salts that are reduced to diformazans as a result of oxidative enzymatic activity. In the present study, neotetrazolium was used, the diformazan of which is a dark purple precipitate easily visualized with the light microscope.

For the demonstration of vaginal mucins, the periodic acid-Schiff (PAS) reaction was employed after tissue fixation in Rossman's fluid and salivary digestion of the tissue sections. Nuclear staining was effected with hematoxylin following the PAS reaction. Although the PAS technique cannot be regarded as a specific tool for demonstrating mucins (Leblond *et al.*, 1957), staining of mucigenous material in the vaginal epithelium was selective and proved satisfactory for histochemically ascertaining the degree of vaginal mucification under any given treatment.

Observations and Results

OXIDATIVE ENZYME SYSTEMS

Following Ovariectomy

Ten days after ovariectomy the vaginal epithelium was reduced from a thickness of 8 to 10 cell layers to a thickness of 4 to 5 cell layers. Coincident with this change was the infiltration of the epithelium with leukocytes, mostly neutrophils. The DPN system activity of such vaginas was low (FIGURE 1a) and closely resembled that of diestrous vaginas (Rosa and Velardo, 1959). A few scattered diformazan granules were deposited within some epithelial cells, but many cells were unreactive. No staining was observed in vaginas of castrated female rats studied for SDH system content.

Treatment with Estradiol-17 β

DPN-diaphorase. Most of the vaginas studied for DPN after estradiol treatment are illustrated in FIGURE 1. It may be seen that daily doses of estradiol as low as 0.005 μ g. had a marked effect upon the DPN activity (FIGURE 1b). In the germinative layers, where the activity was extremely high, all cellular detail was obscured by intense deposits of diformazan. Staining was less intense in superficial cell layers. Vaginas of rats treated with 0.01 μ g. estradiol (FIGURE 1c) also reacted strongly, but a shift in the intensity of activity from basal to luminal layers was evident. This shift in DPN activity was even more conspicuous in the animals receiving 0.025 μ g. where the localization of enzymatic activity in the luminal cells resembled that found during proestrus (Rosa and Velardo, 1959), but with a lesser intensity of reaction than the proestrous cells. Activity in the intermediate and basal layers of this series was discernibly less than that found in the group of animals receiving 0.005 μ g. estradiol. With 0.05 μ g. estradiol, approximately 50 per cent of the vaginas were mucified, whereas the remainder were in a cornified state. Both types of vaginas showed a general drop in enzymatic activity compared to that of the three previous groups. In the mucified vaginas (FIGURE 1d) most of the DPN activity was located in the luminal and germina-



FIGURE 1. Localization of DPN activity in vaginas of nontreated castrated and estradiol- 17β -treated ovariectomized rats. The vaginal lumen is oriented toward the top of the photomicrograph, and the stratum germinativum and subepithelial connective tissue is at the bottom. All tissue sections are $30\ \mu$ in thickness without any counterstaining. $\times 750$. The same conditions, such as of tissue thickness, were used in specimens shown in FIGURES 2 to 6. (a) Vagina of an adult albino rat 10 days after ovariectomy is apparent. (b) Vagina of ovariectomized rat treated with $0.005\ \mu\text{g}$. estradiol. Note heavy deposits of diformazan in the germinative layer of vagina as a result of this low hormone level. (c) Vagina of ovariectomized rat treated with $0.01\ \mu\text{g}$. estradiol. Diformazan is deposited very heavily in the luminal layer of cells. (d) Vagina of ovariectomized rat treated with $0.05\ \mu\text{g}$. estradiol. Note the activity of the luminal, mucified layer of cells. (e) Vagina of ovariectomized rat treated with $0.10\ \mu\text{g}$. estradiol. Upper left area of photograph reveals inactive, cornified vaginal epithelial cells. (f) Vagina of ovariectomized rat treated with $0.5\ \mu\text{g}$. estradiol. Note increased activity over vagina in e.

tive layers, while cells in the intermediate layer appeared to be undergoing initial changes leading to cornification. The cornified vaginas (0.05 μg . estradiol) contained no activity within the luminal cornified cells; activity was greatest in the germinative layer. Vaginas of the 0.1 μg . estradiol-treated rats showed a high degree of cornification, and the completely keratinized cells were unreactive (FIGURE 1e). An intense reaction appeared in the stratum germinativum paralleling progressively decreasing activity in the intermediate layer as cornification advanced. At the level of 0.5 μg . the vaginas appeared to change in one respect only: the intermediate cells contained greater amounts of diformazan (FIGURE 1f) than those found in the former group. At this dosage, cornification of the vaginas was quite pronounced. Animals treated with 1.00 μg . of estradiol had vaginas that had undergone a very high degree of cornification. Localization of DPN within these tissues was quite similar to that shown in FIGURE 1f, although there was an indication of a slight drop in activity.

Succinic dehydrogenase. No SDH activity was found in vaginas of estradiol-treated ovariectomized animals at hormone levels lower than 0.025 μg . FIGURE 2a shows the feeble enzyme activity with this minimal effective dose. Treatment with 0.05 μg ., which produced two main types of vaginas, mucified and cornified, evoked a moderate degree of diformazan deposition. The mucified vaginas (FIGURE 2b) were active in the luminal and germinative layers; in cornified vaginas (FIGURE 2c) activity was absent in completely keratinized luminal cells. Higher amounts of estradiol resulted in a progressive increase in SDH activity, most of this being localized in the basal cell layers and lower portion of the intermediate zone (FIGURE 2d, e, f).

Treatment with Progesterone

DPN-diaphorase. The DPN activity in vaginas of progesterone-treated ovariectomized rats was found to be very variable, depending upon the amount of hormone given (FIGURE 3). The lowest dosage of progesterone (0.05 mg.) induced almost no change in diformazan deposition over the control level of the untreated ovariectomized rats, although the reddish coloration of the background definitely increased over that seen in untreated castrates (compare FIGURES 1a and 3a). The 0.10-mg.-treated rats had vaginas that displayed a very strong reaction, particularly in the luminal mucified layers of the epithelium (FIGURE 3b). This response was increased progressively in vaginas of animals in the 0.25-mg. and 0.5-mg. groups. In the latter group so much diformazan was deposited throughout all cell layers that the morphologic details of staining could not be observed clearly. Vaginas of rats treated with 0.75 mg. showed a slight drop in activity (FIGURE 3c), and this became more evident at the level of 1.00 mg. (FIGURE 3d). At these relatively high doses a general trend of decreasing DPN activity with increasing hormone concentration became most evident at progesterone concentrations of 1.50 mg. (FIGURE 3e) and 4.5 mg. (FIGURE 3f). Although this study was concerned primarily with the enzymatic activity of the vaginal epithelium, it is interesting to note that corresponding changes of DPN activity occurred within fibroblasts of the subepithelial connective tissue; their activity seemed to parallel the diformazan content of the epithelium.



FIGURE 2. Localization of SDH activity in vaginas of estradiol- 17β -treated ovariectomized rats. (a) Vagina of ovariectomized rat treated with $0.025\ \mu\text{g}$. estradiol. Note complete absence of SDH activity from the luminal, mucified layer of cells. (b) Vagina of ovariectomized rat treated with $0.05\ \mu\text{g}$. estradiol. Note SDH activity of the mucified layer and stratum germinativum. (c) Same treatment as b, but resulting in a cornified vagina. Activity is absent from the cornified cells (*upper right*). (d) Vagina of ovariectomized rat treated with $0.10\ \mu\text{g}$. estradiol. The increased cornification of cells results in inactive luminal and intermediate layers (*top of photomicrograph*). (e) Vagina of ovariectomized rat treated with $0.50\ \mu\text{g}$. estradiol. The thickness of the vaginal epithelium is so increased that only the stratum germinativum and lower portion of the intermediate layer are shown. All other components are negative. (f) Vagina of ovariectomized rat treated with $1.0\ \mu\text{g}$. estradiol. Note strong reactivity of many of the cells in the germinative layer. Intermediate (*top*) and luminal layers are unreactive.



FIGURE 3.

Succinic dehydrogenase (SDH). The SDH activity of vaginas of rats treated with progesterone did not show any significant differences. Photomicrographs in FIGURE 4 (most of which have been photographically "overprinted" to bring out structure) indicate no apparent change in enzymatic activity, regardless of the concentration of progesterone administered.

Treatment with Estradiol-17 β and Progesterone

DPN-diaphorase. The DPN stainability of vaginas of animals given a constant dose of estradiol-17 β combined with different amounts of progesterone is shown in FIGURE 5. The activity of the first series (0.10 μ g. estradiol plus 0.05 mg. progesterone) was low (FIGURE 5a). Although a pronounced thickening of the epithelium over that of untreated castrated rats (FIGURE 1a) was evident, the activity of these vaginas was lower than that of those treated with 0.10 μ g. estradiol alone (FIGURE 1e). With increasing progesterone levels (0.25 mg., 0.50 mg.), diformazan deposition was markedly augmented, approaching that obtained with progesterone alone. It is also interesting to note that FIGURE 5c presents a picture quite similar to that of proestrous vaginas studied for DPN activity. A pronounced drop in activity was observed in the vaginas of rats treated with 0.10 μ g. estradiol plus 1.00 mg. progesterone (FIGURE 5d). The staining was significantly less than that seen with either 0.10 μ g. estradiol or 1.0 mg. progesterone (FIGURES 1e and 3d, respectively). Activity was regained, however, with higher doses of progesterone (FIGURE 5e and FIGURE 5f).

Succinic dehydrogenase (SDH). FIGURE 6 illustrates the SDH activity of vaginas of rats after conjoint administration of estradiol and progesterone. The vaginal epithelium of castrated rats given 0.10 μ g. estradiol with 0.05 mg. (FIGURE 6a) and with 0.25 mg. progesterone (FIGURE 6b) exhibits minimal SDH activity, being lower than that found in animals treated with 0.10 μ g. estradiol alone (FIGURE 2d). When the amount of progesterone was raised to 0.5 mg., a very definite increase in SDH was found (FIGURE 6c). As the concentration of progesterone was raised progressively, there was an apparently poor correlation between higher dosage and greater SDH activity. Increasing the amount of progesterone to 1.0 mg. resulted in a drop in activity (FIGURE 6d) over the previous series (FIGURE 6c), but a moderately strong reaction was elicited in vaginas after treatment with 1.5 mg. progesterone plus 0.1 μ g. estradiol (FIGURE 6e). Addition of 4.5 mg. of progesterone to the estradiol treatment, however, resulted in lowered activity (FIGURE 6f), which cor-

FIGURE 3. Localization of DPN activity in vaginas of progesterone-treated ovariectomized rats. (a) Vagina of ovariectomized rat treated with 0.05 mg. progesterone. Progesterone at this level appears to have some effect on the DPN activity of vaginas of ovariectomized rats. Compare with FIGURE 1a. (b) Vagina of ovariectomized rat treated with 0.10 mg. of progesterone. Note heavy activity in vaginal epithelium and connective tissue fibroblasts. (c) Vagina of ovariectomized rat treated with 0.75 mg. progesterone. Compare with b and d. Note desquamating cell (top). (d) Vagina of ovariectomized rat treated with 1.0 mg. progesterone. Note decreased activity over that seen at the level of 0.75 mg. (c). (e) Vagina of ovariectomized rat treated with 1.5 mg. progesterone. Photomicrograph is slightly overprinted to bring out morphology. Pink staining with occasional granules of diformazan characterizes vagina of rats treated at this level of progesterone. (f) Vagina of ovariectomized rats treated with 4.5 mg. progesterone. Reaction of the vagina is similar to that seen in e despite a slight proliferative response of the epithelium at this higher level.



FIGURE 4. Localization of SDH activity in vaginas of progesterone-treated ovariectomized rats. Photographs *a*, *b*, *c* and *e* are overprinted to bring out morphology. (*a*) Vagina of ovariectomized rat treated with 0.05 mg. progesterone. Extremely pale pink staining prevails. A granule of diformazan is observed rarely. (*b*) Vagina of ovariectomized rat treated with 0.10 mg. progesterone. Staining is low, but diformazan in the form of granules can be seen in sparse amounts. (*c*) Vagina of ovariectomized rat treated with 0.25 mg. progesterone. Activity is similar to that in the previous photograph. (*d*) Vagina of ovariectomized rat treated with 1.0 mg. progesterone. Although appearing to demonstrate less activity than *b* and *c*, this print shows the true activity present at this level of progesterone. Microscopically, vaginas as in *b* and *c* can not be distinguished from this preparation. (*e*) Vagina of ovariectomized rat treated with 1.5 mg. progesterone. Activity is very low in vaginas at this level of progesterone treatment. (*f*) Vagina of ovariectomized rat treated with 4.5 mg. progesterone. Activity here is comparable to that seen in *e*.

responded to the reaction given after treatment with progesterone alone (FIGURE 4).

VAGINAL MUCINS

Following Ovariectomy

Mucins were demonstrable in the vaginas of rats ten days after ovariectomy. Most of the PAS-positive material, however, was found to be extracellular in location. A layer of mucin approximately one cell in thickness existed in contact with the superficial layer of epithelial cells (FIGURE 7a). It was not possible to ascertain whether this superficial layer of mucin was of vaginal or cervical origin. In addition, irregular intracellular deposits of mucin occasionally were seen in the epithelium. This appeared mostly in the surface cells, where it imparted a light granular staining within the cytoplasm of these reactive cells. An accumulation of such material into large deposits was a rare feature seen in many of the vaginas studied.

Treatment with Estradiol-17 β

Animals treated with 0.005 μ g. of estradiol had low, yet significantly higher deposits of PAS-staining material in the vaginal epithelium, with stainable substance more restricted to superficial layers than had untreated castrates (FIGURE 7b). Very little if any cellular proliferation, however, was induced as a result of this low dose of estrogen. Some growth was apparent, however, when the dosage of estrogen was increased twofold (0.01 μ g.), and more frequent deposits of increased mucin *within* the epithelium were seen (FIGURE 7c). The general pattern of distribution for the stainable material was approximately the same here as for the untreated castrated rats and the estrogen-treated animal with lower levels of hormone. A dosage level of 0.025 μ g. induced appreciable deposition of PAS-positive material (FIGURE 7d). Mucin, obscuring cellular details of the superficial layers, was seen discretely within these cells. With 0.05 μ g. there appeared a definite increase in the thickness of the epithelium, estimated at about double that found with the 0.025 μ g. level. The vaginal response with 0.05 μ g. estradiol was separable into two categories. Approximately one half of the animals contained cornified vaginal epithelium with no demonstrable mucification (FIGURE 7f). The remainder of the animals showed considerable mucification in the superficial cell layers. Neither the intermediate nor germinative layers displayed any PAS-positive material. In the former, cytomorphological changes suggestive of incipient cornification were evident (FIGURE 7e). All animals receiving dosages of estrogen higher than 0.05 μ g. bore vaginas that were highly cornified and completely devoid of mucin in all epithelial layers.

Treatment with Progesterone

Despite variations in vaginal mucification, proliferative activity within the vaginal epithelium was consistently absent after progesterone treatment. Occasionally, some slight increase in thickness of the vagina over that of the untreated castrated rat was observed, but in most animals the vaginal histology resembled that of the untreated ovariectomized controls.

Both extra- and intracellular deposition of PAS-positive material was found in castrates administered 0.05 mg. of progesterone (FIGURE 8a). Most of the mucin was deposited within the vaginal lumen directly above the superficial layer of cells. Staining *within* cells was feeble and restricted mostly to the first cell-layer zone. Increasing the dosage of progesterone from 0.05 to 0.10 mg. resulted in intense staining of the cells of the superficial zone and cells exfoliated into the lumen of the vagina (FIGURE 8b). Intracellular deposits of mucin could be seen in cells that showed no apparent change in size. In addition, the amount of stainable material found within the lumen was increased considerably when compared with nontreated or 0.05-mg. progesterone-treated castrate vaginas. Mucification decreased at 0.25-mg. (FIGURE 8c) and 0.50-mg. (FIGURE 8d) dose levels, but was again intense at higher dose levels tested, such as 0.75 mg. (FIGURE 8e) and 1.00 mg. (FIGURE 8f).

Treatment with Estradiol-17 β and Progesterone

A strong degree of cornification ensued in vaginas with 0.10 μ g. estradiol and 0.05 mg. progesterone (FIGURE 9a). It should be noted that dosages of progesterone of 1.0 mg. or less in conjunction with 0.10 μ g. estradiol did not interfere seriously with the growth-promoting effects of 0.10 μ g. estradiol on the vaginal epithelium, but higher doses inhibited epithelial growth effectively (FIGURES 9e and 9f).

The 0.25-mg. dose level, while inhibiting cornification (but not proliferation) resulted in considerable deposition of both intracellular and extracellular mucins (FIGURE 9b). A few exfoliated cells were found in close proximity to the well-stained superficial layer of cells. Such cells contained voluminous amounts of cytoplasmic PAS-positive material, which in many instances obscured nonreactive cytoplasmic constituents. Mucinogenesis and exfoliation were maximal in vaginas from castrated rats treated with 0.10 μ g. estradiol plus 0.50 mg. progesterone (FIGURE 9c). These processes subsided somewhat when the progesterone dosage was increased to 1.0 mg. (FIGURE 9d). The vaginal epithelium was well infiltrated with leukocytes concentrated in the intermediate and superficial layers. These blood cells contained PAS-positive material, removable with salivary digestion, suggesting the presence of glycogen. The degree of mucification resembled its former height of production at the 1.50 mg. level (FIGURE 9e), but 4.50 mg. progesterone in conjunction with 0.10 μ g. estradiol (FIGURE 9f) tended to induce a lesser state of mucification.

FIGURE 5. Localization of DPN activity in vaginas of ovariectomized rats treated with 0.1 μ g. estradiol-17 β plus progesterone. (a) Vagina of ovariectomized rats treated with 0.10 μ g. estradiol plus 0.05 mg. progesterone. Low activity is present and almost entirely confined to the stratum germinativum. Compare with FIGURES 1a, 1e, and 3a. (b) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 0.25 mg. progesterone. Note the intense activity in the germinative and intermediate layers. (c) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 0.50 mg. progesterone. Proliferation of the vagina is evident, and not all of the basal layers of cells are shown. These germinative cells are very active, as are also the luminal (*top*) mucified cells. (Compare with FIGURE 9c.) (d) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 1.0 mg. progesterone. Note the decided drop in activity in this picture as compared with that of c and e. (e) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 1.5 mg. progesterone. Moderate to heavy activity prevails in most cells of the vaginal epithelium. (f) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 4.5 mg. progesterone. Very intense activity is localized in the luminal and basal layers, whereas cells in the intermediate area show less activity.



FIGURE 5.

Discussion

OXIDATIVE ENZYME SYSTEMS

To establish a base line of vaginal epithelium activity, adult ovariectomized rats were tested histochemically for SDH and DPN. Under these conditions of study, SDH activity had completely disappeared from the vaginal epithelium 10 days after operation. On the other hand, some DPN activity remained that resembled the diformazan pattern found during diestrus in previous studies (Rosa and Velardo, 1959). Whether these low activities of the vagina are stabilized at this level or continue to decline beyond the 10-day period of ovariectomy was not determined.

The results obtained by injecting castrated rats with graded doses of estradiol-17 β show that both DPN and SDH activity can be restored and activated above normal levels found in the vaginal epithelium. An obvious difference is the sensitivity of response of SDH compared with that of DPN. The DPN system is capable of responding to extremely low amounts of estradiol (0.005 μ g.), while it requires at least 5 times this amount of hormone to invoke even a feeble SDH response in the vagina after castration. Moreover, DPN activity can be augmented markedly with very low dosages of estradiol, which have been shown to elicit the production of mucin (*see below*). Thus, DPN activity possibly could be associated with mucin formation under these experimental conditions. At approximately 10 times the minimal effective dose for DPN activity, mucified and cornified vaginas appear in a 50:50 ratio, suggesting that this dosage is the threshold level for initiating vaginal cornification. There appears to be a drop in enzymatic activity at this level; although the mechanism underlying this decreased DPN activity is unknown, it is possible that this reflects a shift in metabolic processes related to the type of protein synthesized (that is, mucoprotein versus keratin). Higher concentrations of estradiol (0.10 to 1.0 μ g.) again increase the activity of DPN, especially in the basal and intermediate cell layers, but the keratinizing cells progressively lose enzymatic activity, and this presumably may be related to decreased metabolism prior to cell death.

The vaginal SDH activity of estradiol-treated rats apparently is related directly to increasing concentrations of this growth-promoting, mitogenic hormone. As mentioned previously, however, much higher concentrations of

FIGURE 6. Localization of SDH activity in vaginas of ovariectomized rats treated with 0.1 μ g. estradiol plus progesterone. (a) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 0.05 mg. progesterone. Enzymatic activity is extremely low in this vagina. All activity is restricted to the germinative layers. Darkening of the photograph in other regions is due to the density of the cells in these areas. (b) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 0.25 mg. progesterone. Here again, as in *a*, all diformazan deposition occurs in the basal layers. Note lightly increased activity over that seen in *a*. (c) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 0.50 mg. progesterone. This cornified vagina (*upper right*) shows a moderate to strong reaction in the stratum germinativum with lesser activity in the intermediate layer of cells. (d) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 1.0 mg. progesterone. A drop in activity is evident in the germinative and intermediate layers when compared with *c*. (e) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 1.5 mg. progesterone. An increased amount of diformazan in this vagina over that in *d* can be seen. (f) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol plus 4.5 mg. progesterone. Note the precipitous drop in activity in this vagina as compared with that shown in *e*.

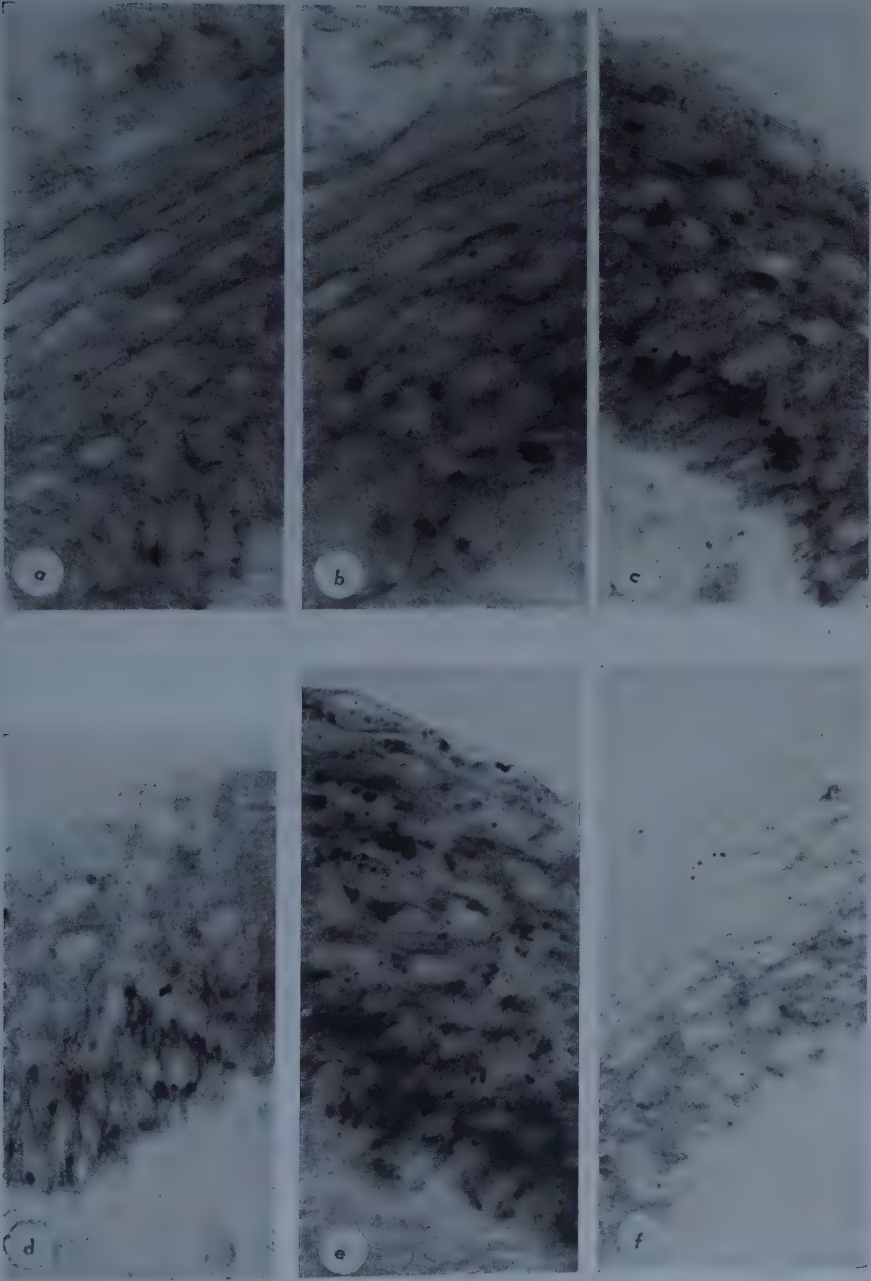


FIGURE 6.

hormone are required to evoke reasonably good diformazan deposition. This basic difference between the relative sensitivities of SDH and DPN under the influence of estradiol might be related to the intracellular distribution of these enzymes. Perhaps the succinate system that is primarily associated with mitochondria (Schneider, 1955) may be less readily accessible to hormonal influence, in contrast to DPN activity as studied in this work, the lactic dehydrogenase component of which is believed to be in the soluble fraction of the cell (Le Page and Schneider, 1948; Hers *et al.*, 1951). It is interesting to note that high sensitivity of the DPN system to estrogen has been observed in studies of the uterus of castrated rats as determined in homogenates (Bever, 1959).

In vaginas of ovariectomized rats given different amounts of progesterone, wide differences between SDH and DPN activity were found. The doses of progesterone used caused little, if any, proliferation in the vaginas of castrated rats. The lack of mitotic activity and the almost complete absence of SDH activity suggest an association between growth and increased Krebs cycle activity, an idea already proposed for this general type of epithelial structure (Bullough, 1952).

On the other hand, it has been demonstrated that the DPN system is activated independently of growth phenomena. In contrast, there is good correlation between mucin production and high DPN activity. The DPN activity in vaginas treated with progesterone was not found to be related directly to hormone concentration. With 3 doses daily of 0.50 mg. progesterone, a peak reaction was obtained that declined rapidly with either an increase or a decrease in progesterone dosage. We are at present unable to explain this phenomenon; a somewhat similar situation exists in the lactic dehydrogenase activity of uteri in progesterone-treated rats in spite of the fact that DPNH-oxidase activity is low at the 0.50-mg. level of progesterone (Bever *et al.*, 1953a; Bever, 1959).

With combined treatments of 0.10 μ g. estradiol-17 β and progesterone, SDH and DPN activity varied considerably. In addition, the degree of

FIGURE 7. The distribution of vaginal mucins in ovariectomized and estradiol-17 β -treated ovariectomized rats as demonstrated by the PAS reaction after salivary digestion in tissue sections 7 μ in thickness. Hematoxylin-counterstained. $\times 750$. (a) Vagina of an adult albino rat 10 days after ovariectomy. Extracellular mucin, approximately one cell thick, caps the epithelium. Note the presence of some intracellular PAS-reaction material in the superficial layer of cells, especially at the left. (b) Vagina of an ovariectomized rat treated with 0.005 μ g. estradiol. This very low dosage of hormone induces some increase in the mucin production of the luminal cells. (c) Vagina of an ovariectomized rat treated with 0.01 μ g. estradiol. Increased thickness in the epithelium is apparent and intracellular deposits of mucin can be seen clearly. The vagina shown contains lesser amounts of stainable material than most others in this group, but it demonstrates better the intracellular material in the absence of obscuring amounts of extracellular mucin. (d) Vagina of ovariectomized rat treated with 0.025 μ g. estradiol. More stainable material can be seen in the superficial layers of cells compared to that seen in a to c. Heavy deposits within cells make their appearance for the first time at this level of treatment. (e) Vagina of ovariectomized rat treated with 0.05 μ g. estradiol, demonstrating a most marked degree of mucification. This is the greatest amount of mucin formation induced by estradiol prior to cornification. Here it can be seen that more than one cell layer is involved in mucin synthesis. The epithelial thickness indicates considerable proliferative activity. (f) Vagina of ovariectomized rat treated with 0.05 μ g. estradiol, demonstrating cornification. This photomicrograph shows the degree of cornification (*top*) induced by such a dosage. Proliferation here is similar to that in e.



FIGURE 7.

mucification and/or cornification also varied markedly. Such conditions of wide variability may be the result of individual differences in dose response, in turn a reflection of induced fluctuations in the titer of adrenocortical steroids. Indications that the latter can be operative on both a physiological and enzymatic basis have been shown by Velardo *et al.* (1953a, 1956) and Bever *et al.* (1953b), respectively.

These histochemical observations on the SDH and DPN activity of the conjoint hormone treatments have shown that conditions inducing the production of mucin in castrate vaginas also increased DPN activity. On the other hand, growing and cornifying vaginas appear to utilize both SDH and DPN in the production of phosphoric esters of the high-energy variety. The relative intensities of enzymatic activity obtained with various levels of progesterone added to the standard 0.10 μ g. estradiol dosage make it possible to attempt an interpretation of these results in terms of competitive and synergistic hormonal activity as related to the oxidation of a particular substrate (that is, lactate or succinate).

In the following analysis, results obtained with 0.10 μ g. estradiol and progesterone separately and in combination will be compared with each other. Enzymatic activity after treatment with a single hormone will be considered as the comparable, control level. When such comparisons are evaluated it becomes evident that, in the case of DPN, 0.05 mg. of progesterone inhibits the action of 0.10 μ g. estradiol, as does also 1.00 mg. of progesterone. Synergism of estradiol and progesterone with respect to this enzyme system is apparent at the 0.25- to 0.50-mg. and the 1.5- to 4.5-mg. levels of progesterone, respectively.

Synergisms and antagonisms are also evident in the SDH activity of these vaginas, although some quantitative differences in dose response by SDH and DPN do occur. Thus, the levels of 0.05 to 0.25 mg., 1.0 mg., and 4.5 mg. progesterone, respectively, inhibit the SDH activity that would have been evoked by 0.10 μ g. estradiol treatment alone. Conversely, these hormones synergize at the 0.50-mg. and the 1.5-mg. levels, respectively (TABLE 1).

VAGINAL MUCINS AND OXIDATIVE ENZYMES

The effects of estradiol-17 β on the vaginal epithelium in the rat are twofold. Dosages at 0.05 μ g. or below can induce the secretion of PAS-positive material.

FIGURE 8. The distribution of vaginal mucins in progesterone-treated, ovariectomized rats. The technique used and the magnification are similar to those in FIGURE 7. (a) Vagina of ovariectomized rat treated with 0.05 mg. progesterone. The presence of some discrete staining material can be seen. Most PAS-positive material is finely dispersed, highly granular, and localized mainly in the superficial cell layers. Extracellular mucin is not shown. (b) Vagina of ovariectomized rat treated with 0.10 mg. progesterone. Note increased staining of the superficial cells. (c) Vagina of ovariectomized rat treated with 0.25 mg. progesterone. Mucin staining is still present in the outer zone of cells, but some decrease is evident as compared with that seen in b. (d) Vagina of ovariectomized rat treated with 0.50 mg. progesterone. The decreased staining seen in the previous level (c) is further restricted in vaginas of rats treated with 0.50 mg. progesterone. Very narrow deposits are seen in the luminal epithelium, most mucin being extracellular. (e) Vagina of ovariectomized rat treated with 0.75 mg. progesterone. Note the increased deposits of mucin compared to the former (d) lower dosage. (f) Vagina of ovariectomized rat treated with 1.0 mg. progesterone. The trend for increased mucin production at 0.75 mg. progesterone and above is more obvious here. Deposits of PAS-positive material are higher than those seen in e.

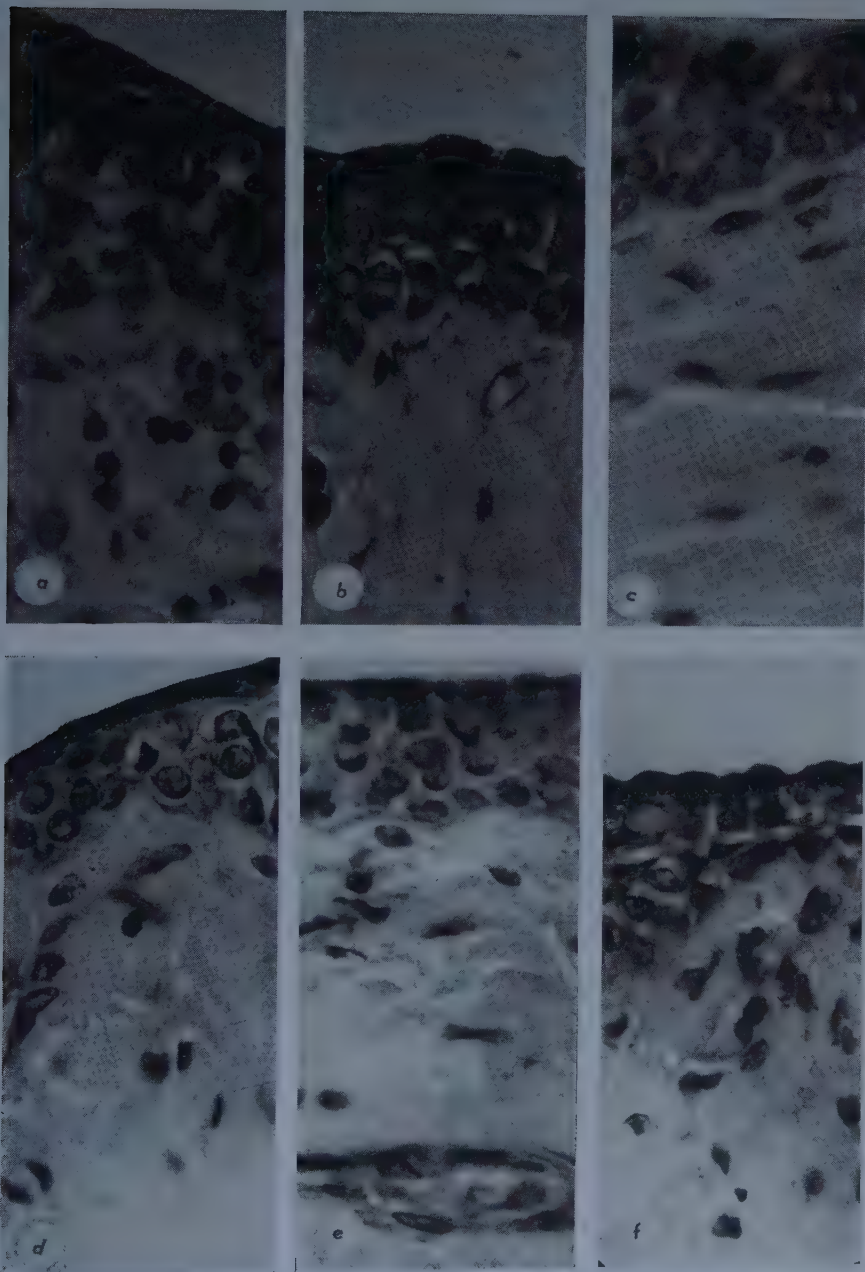


FIGURE 8.

Apparently, increasing concentrations of this estrogen within these limits of hormone dosage progressively increase the amount of mucin produced in the superficial cell layers. Such effects have been recognized for some time (Meyer and Allen, 1933), although some factors, such as the conditions of treatment employed by other workers were somewhat different from those reported in the present work. The 0.05- μ g. estradiol level in our experiments is evidently a threshold level, since some animals contain mucified vaginas while others have a well-cornified vaginal epithelium. Higher doses of estradiol always resulted in cornified vaginal epithelium and increased proliferation of this structure. If one relates these phenomena to the oxidative enzyme systems, a fairly good correlation can be seen between proliferative activity (mostly found in the lower layers of the epithelium) and both SDH and DPN activity. The latter enzyme system, however, is apparently activated independent of growth. Generally, it seems that where there is such proliferation, good SDH activity is found, although this enzyme system appears to be less associated with mucification processes in comparison to DPN.

Vaginas from progesterone-treated castrated rats reveal similar correlations. Little or no growth was ever found in such vaginas, and the SDH localizations showed a striking lack of oxidative enzymatic activity as a result of this particular system. Mucification or mucin synthesis, however, was visualized under such experimental conditions with comparatively high DPN activity. The roughly straight-line relationship for mucin synthesis which holds for increasing hormone at low levels (low) of estradiol was not obtained with progesterone. With the latter, some decrease in PAS-positive material is found at the 0.10-mg., 0.75-mg., and 1.00-mg. dose levels. This situation is comparable to uterine enzymatic phenomena concerned with the lactic dehydrogenase-DPNH oxidase system (Bever, 1959).

Correlations similar to the above considerations also were seen in the estrogen-

FIGURE 9. The distribution of vaginal mucins in estradiol- and progesterone-treated ovariectomized rats. The technique used and the magnification are similar to those of FIGURES 7 and 8, except for *d*, which was not subjected to salivary digestion. (a) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol and 0.05 mg. progesterone. This photomicrograph demonstrates the high degree of cornification induced by such estrogen treatment and the inability of this level of progesterone to inhibit estrogen-induced cornification. The cornified vaginal epithelial cells are located at the top of the photomicrograph and are out of focus, since they lie out of the plane of the lower layers of epithelium. Note also that the cornified elements are in the process of desquamating from the adjacent precornified cells. Considerable vaginal epithelial proliferation is apparent. (b) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol and 0.25 mg. progesterone. Cornification is inhibited here by this level of progesterone, and much PAS-positive material can be seen in the luminal cell layers. (c) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol and 0.50 mg. progesterone. Note the high degree of mucification and exfoliation of the superficial cells. (d) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol and 1.0 mg. progesterone. A decrease in mucinogenesis and exfoliation is evident when compared with *f*. The dark granular elements in the intermediate cell layers represent blood leukocytes. The stainable material within such cells is probably glycogen, since salivary digestion prior to the PAS reaction removes this substance. (e) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol and 1.5 mg. progesterone. Mucin formation is slightly increased at this level. Some exfoliated cells are apparent on the top of the superficial cells. Note that these appear to contain less mucin than similar cells seen in *c*. Thickness of epithelium is somewhat decreased compared to previous treatments. (f) Vagina of ovariectomized rat treated with 0.10 μ g. estradiol and 4.5 mg. progesterone. A decided drop in mucin synthesis is evident, and exfoliation is practically absent. Note decrease in epithelial thickness, indicating progesterone inhibition of the cell proliferative effects of estradiol.

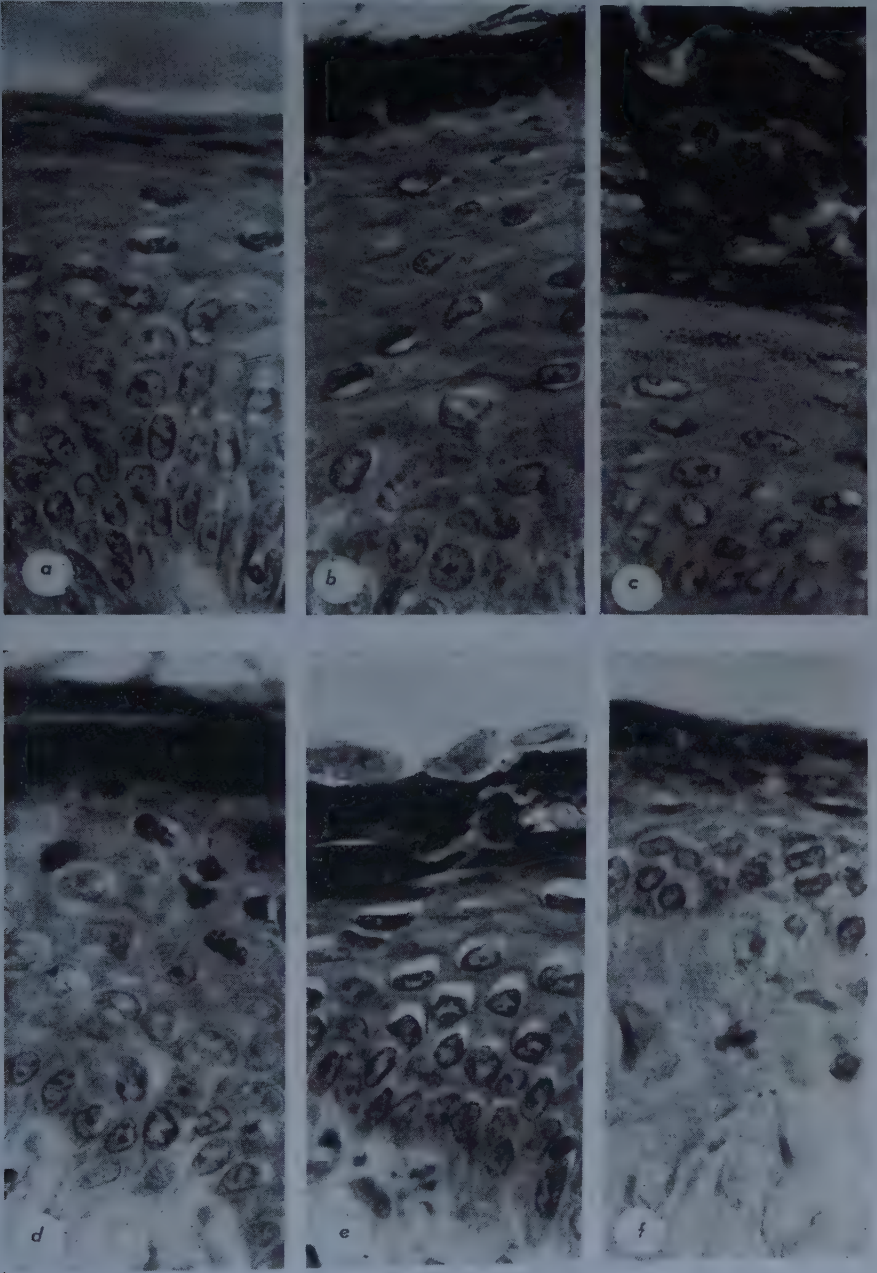


FIGURE 9.

plus-progesterone series where, in many instances of high mucin localization, corresponding high degrees of DPN activity could be found. It should be recalled also that, when pronounced proliferation was manifest, high SDH activity also prevailed. However, detailed examination of each treatment and the results obtained with mucin, DPN, and SDH localizations do not always

TABLE 1
LOCALIZATION AND INTENSITY OF VAGINAL HISTOCHEMICAL REACTIONS

Treatments	SDH			DPN			Mucification		
	L	I	B	L	I	B	L	I	B
Castrated rat (10 days)	0	0	0	+	+	+	+ or 0	0	0
Estradiol									
0.005	0	0	0	++	++	++++	+	0	0
0.01	0	0	0	++++	++++	++	++	0	0
0.025	0	+	+	++++	+	++	+++	0	0
Mucification	++	+	++	++	+	++	++++	0	0
0.05									
Cornification	0	+	++	0	+	++	0	0	0
0.10	0	+	++	0	+++	++++	0	0	0
0.50	0	++	++	0	++++	++++	0	0	0
1.0	0	+++	+++	0	+++	++++	0	0	0
Progesterone (mg.)									
0.05	0	0	+	++	++	++	+	0	0
0.10	+	+	+	++++	++++	++++	+++	+	0
0.25	+	+	+	++++	++++	++++	++	0	0
0.50	+	+	+	++++	++++	++++	+	0	0
0.75	+	+	+	++++	++++	++++	++	0	0
1.0	+	+	+	+++	++++	+++	+++	0	0
1.50	+	+	+	++	++	++	not studied not studied		
4.50	0	+	+	0	+	+			
Estradiol (0.10) plus progesterone (mg.)									
0.05	0	+	+	0	+	++	0	0	0
0.25	0	+	++	+	++	++++	++++	0	0
0.50	0	+	++	++++	+++	++++	++++	0	0
1.0	0	+	+	+	++	+++	++++	0	0
1.50	+	+	++	+++	+++	++++	++++	0	0
4.5	0	0	+	++++	+++	++++	+++	0	0

Key: L, luminal layers; I, intermediate layer; B, basal layer; 0, none; +, low; ++, moderate; +++, high; +++++, very high.

show complete correlation with the generalizations suggested above. There are instances when DPN is lower than would be expected (0.05 μ g. estradiol) or when PAS-positive materials are found in amounts not completely corresponding to the DPN activity (0.10 μ g. estradiol and 4.50 mg. progesterone). In any final analysis such exceptions to the general findings obviously must be taken into consideration, but it is not felt that explanations for these inconsistencies can be formulated presently on the basis of these experiments. Some

lack of precision is certainly possible in such a histochemical analysis. It must be understood, in addition, that the experimental conditions actually represent an idealized treatment and that the continuation of such treatments over extended periods of time could yield results of a different order. Our present knowledge is uncertain concerning the temporal sequence of enzymatic events related to mucin synthesis. Which oxidative enzyme systems are stimulated initially, or is there simultaneous stimulation, or indeed, what other enzymes are involved in such reactions? Answers to these and other questions must await further investigation. For the present, it is believed that the study of DPN, and perhaps related enzyme systems, should offer an avenue for the direct approach to such problems. This histochemical analysis, by virtue of the study of tissue sections, has afforded the direct visualization of cell layers in their natural environment. Thus, the identification of a given region within the vaginal epithelium with mucin production and DPN activity has been possible. Therefore, it would appear that an extension of this line of investigation should involve a biochemical approach that would permit more precise quantitation of such mucins and enzymes. Such an endeavor may resolve fruitfully the detailed mechanisms of mucin synthesis and its associated enzymes in reproductive target structures.

Thus, in the final analysis, these results are in harmony with the concept that the several metabolic alterations of the female reproductive tract represent the expression of the sum total of all the hormones and for their metabolites acting in concert. Furthermore, evidence at hand strongly suggests that the hormones and/or their metabolites effect their expression at the molecular level of the different cell types comprising the endocrine receptor organs (Talalay and Williams-Ashman, 1958; Velardo, 1958*a*, 1958*b*, 1959).

Summary

The histochemical localization of the succinic dehydrogenase system (SDH), the diphosphopyridine nucleotide-diaphorase system (DPN), and mucin was determined in the vaginal epithelium of the rat after ovariectomy and following treatment with estradiol and progesterone administered singly or in combinations. Vaginas of nontreated castrated rats contained minimal DPN activity and no histochemically demonstrable SDH. In general, the DPN system appears to respond more readily to hormonal stimulation, whereas SDH activity is mostly low or absent under comparable experimental conditions.

The DPN and SDH systems tend to behave characteristically both in localization and intensity of reaction, depending on the type and dosage of hormone administered. Synergistic and antagonistic reactions are evident when both hormones are administered simultaneously. Both DPN and SDH generally show good reactions in areas of the vaginal epithelium involved in proliferation. Mucin synthesis, on the other hand, appears to be associated with areas containing high DPN activity.

It is suggested that future biochemical experimentation be instigated in order to define more critically some of the underlying mechanisms visualized in these histochemical studies, such as the relationship between DPN activity and mucinogenesis.

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SENSITIVITY OF THE VAGINA TO ESTROGEN: GENETIC AND TRANSMITTED DIFFERENCES

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The estrous cycles, as revealed by examination of the exfoliated cells of the vaginal mucosa of mice of several different inbred strains, differed greatly in length, regularity, and duration of the estrous phase.¹⁻⁶ The first studies were undertaken to determine whether the characteristics of the estrous cycles could be associated with the tendency of mice of the several strains to have different incidences of mammary cancer, after it had been revealed that estrogens played some role in mammary tumorigenesis. Such studies might reveal differences in the exposure of the animals tested to different levels of intrinsic estrogens that might, in turn, be due to differences in vaginal sensitivity to estrogens, in ovarian activity, or in gonadotrophic activity.

Extrinsic estrogens, when injected subcutaneously into ovariectomized mice of different inbred strains, also revealed differences in the responsiveness of the vaginal mucosa in mice of some strains,⁷⁻¹¹ but not between others.³ Non-inbred mice (stock mice), in at least one study, showed a more uniform response to minimal doses of estrogen than did mice of the inbred CBA strain.¹³ Mice of the CBA strain, when studied later in another country, also showed a variable vaginal response to estrogen.¹¹ The strain differences were measured by comparing the minimal dose required to induce a standard minimal response or by determining the relative durations of responses to a standard dose somewhat in excess of the minimal doses. Mice of some strains required more than four times as much estrogen to produce a response of 50 per cent $\pm$ 10 per cent of the test animals. Among mice of some strains the variation between animals was relatively great; among others, very small.^{9, 11} Observations of this type indicated that the vaginal responses of mice of different strains differed, but failed to show (1) whether this was attributable to differences in the rate of inactivation, destruction, or excretion of the hormones injected; (2) whether this could be attributed to differences in the presence in the animals of substances that might antagonize or augment the vaginal response; or (3) whether the vaginal mucosa was actually more or less responsive to estrogen in mice of different strains.

When the steroidal estrogens were applied topically to the vaginal mucosa, much smaller doses induced a response than when they were applied systemically.¹⁴ The same general order of strain differences was revealed after the topical application of estrogen,⁹ an observation that showed that strain differences in vaginal sensitivity probably were not attributable to differences in the capacity to inactivate, destroy, or excrete estrogens because these variables probably were minimized by direct application of the hormone.

Hybrid mice had vaginal sensitivities to injected estrogens that were usually

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intermediate between those of the two parental strains, an observation that indicated a transmission of the tendency that might well be based on multiple factors or influences.¹¹

A method was devised to determine whether the strain differences in vaginal sensitivity were attributable to differences intrinsic to the vaginal tissue. Vaginal tissue from inbred mice of strains with contrasting vaginal sensitivity were transplanted subcutaneously into their hybrid offspring.¹² In this manner

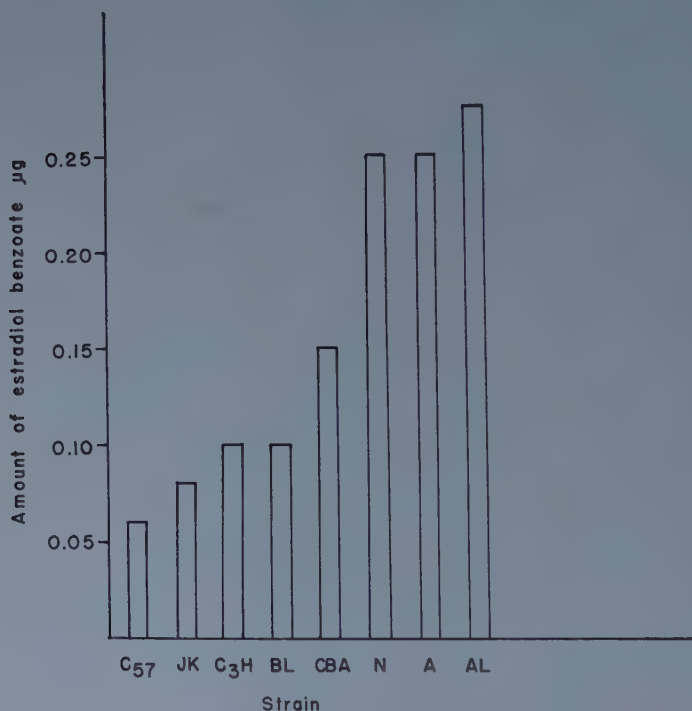


FIGURE 1. The amount of estradiol benzoate required to produce vaginal cornification in 50 per cent $\pm$ 10 per cent of mice of 8 groups (one injection in 0.05 ml. of sesame oil; absence of leukocytes and full cornification required for test).¹¹

tissues from strains showing contrasting vaginal sensitivities to estrogen could be brought into an identical environment.

Mice of the A and C₅₇ strains were selected because they showed not only contrasting sensitivities, but also a relatively narrow range of individual differences in sensitivity within the strain^{11, 12} (FIGURE 1). The hybrid young showed an intermediate vaginal sensitivity to estrogen. The vaginas of donors from 16 to 24 days of age were removed, dissected free from adjacent tissues, and transplanted subcutaneously into the ventrolateral subcutaneous areas of the body of young adult hosts. The subjacent abdominal musculature was scarified slightly to abet attachment and fixation of the graft. The vaginas of the donors at this age were imperforate and, with reasonable care, grafts without bacterial contamination were obtained easily. Care was taken to

exclude the lower uterine horns and cervix, as well as the peripheral vagina. The grafts almost always persisted and grew. They measured up to more than 1 cm. in diameter within 6 weeks to 2 months in many mice that were permitted

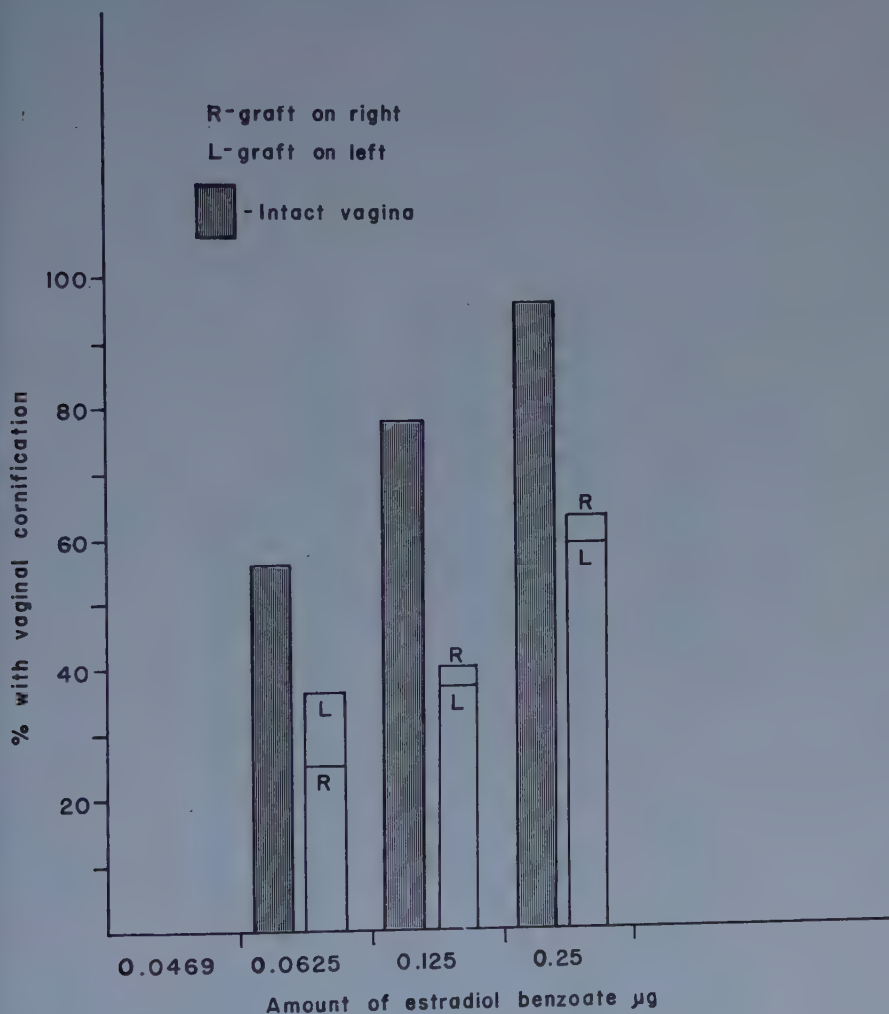


FIGURE 2. Response of intact and transplanted vaginas of mice of the A strain to different amounts of estradiol benzoate. The smallest number of tests at any point was 20 and the largest number was 41 (data from Gardner and Argyris¹²).

to continue normal estrous cycles or that received injections of estrogen. When the grafts became sufficiently large they were opened and sewed to the skin to establish a fistular opening. Smears of the cellular contents were obtained by inserting the small end of a flattened moistened toothpick into the opened fistula. Similar methods were used to obtain exfoliated cells from the intact vagina.

The influence of intrinsic hormones on the intact and transplanted vaginas was studied by daily observation of the smears of exfoliated vaginal cells of the intact animals. Homologous grafts into mice of the A strain responded only about one half as well as did the intact vaginas, whereas homologous

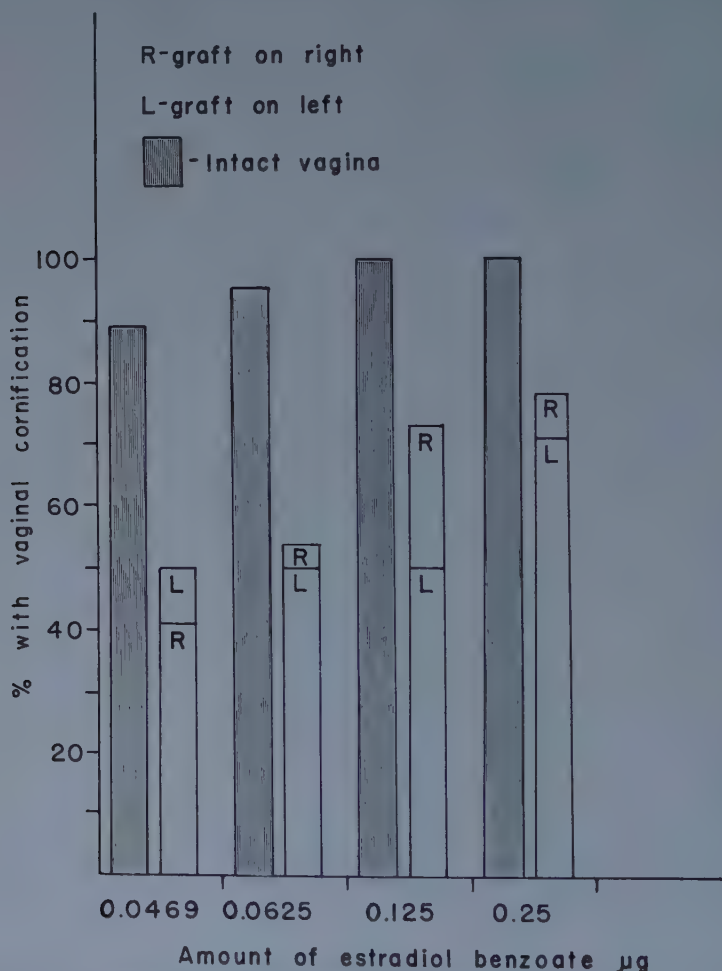


FIGURE 3. Response of intact and transplanted vaginas of mice of the C_{57} strain to different amounts of estradiol benzoate. From 14 to 37 tests were run at each dose level (data from Gardner and Argyris¹²).

grafts into the C_{57} strain and of their hybrid group ($A \times C_{57}$) responded somewhat better. The hybrid hosts provided a more effective environment for the grafts of the A and C_{57} donors than did hosts of the parental strain.

The injection of extrinsic estrogen provided more critical data because comparable doses at reproducible and known levels were assured. Approximately 50 per cent of the intact vaginas from mice of the A strain responded to 0.0625

$\mu\text{g.}$ of estradiol benzoate, but only 25 to 30 per cent of the transplanted vaginas responded. To doses of $0.25 \mu\text{g.}$, 96 per cent of the 28 tests showed cornification of the intact vaginas, whereas 69 to 72 per cent of the transplants showed cornified smears (FIGURE 2).

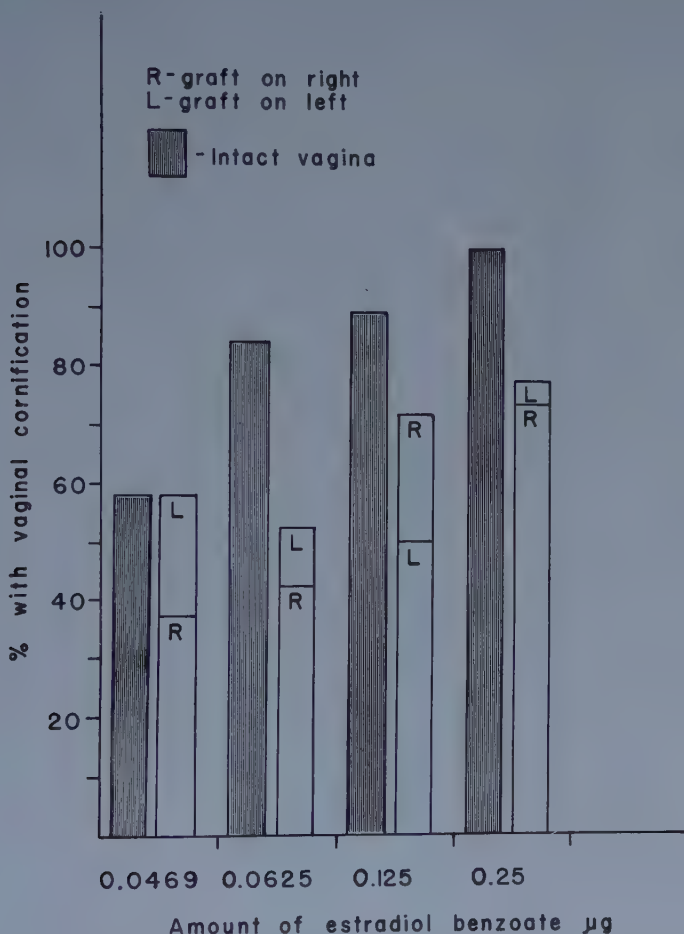


FIGURE 4. Response of intact and transplanted vaginas of hybrid mice ($A \times C_{57}$) to different amounts of estradiol benzoate. From 19 to 38 tests were run at each dose level (data from Gardner and Argyris¹²).

The vaginal cornification of both the intact and transplanted vaginas of mice of the C_{57} strain was significantly more responsive to comparable amounts of estrogen (FIGURE 3) when compared to those of mice of the A strain. From 14 to 37 tests were used to determine each point. The hybrid mice were almost as responsive to injected estradiol benzoate as were mice of the C_{57} parental strain and, again, only about one half of the transplanted vaginas responded to $0.0469 \mu\text{g.}$ of estradiol benzoate (FIGURE 4). The dose was close to the critical dose level because only 11 of the 19 tests showed cornified vaginas. Four of

5 tests conducted with doses of 0.0389 $\mu\text{g.}$ of estradiol also induced vaginal cornification (data from reference No. 12 and not included in FIGURE 4).

The critical tests, however, involved the transplantation of vaginas of A and C_{57} donors into the same hybrid host. A total of 144 tests was undertaken: 126 of the intact vaginas, 93 of the C_{57} -strain vaginas, and 62 of the A-strain vaginas showed vaginal cornification (FIGURE 5). The data summarizing the

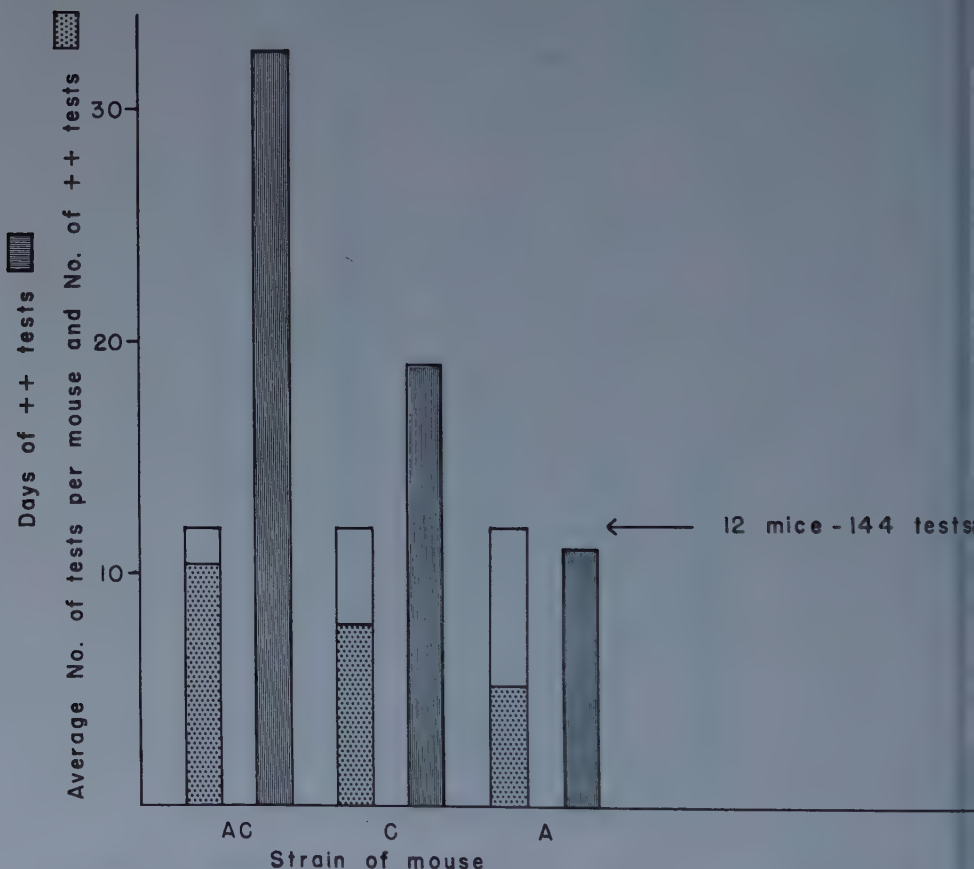


FIGURE 5. Responsiveness of the intact vaginal mucosa of 12 hybrid mice and of the transplanted vaginas from the two parental strains (A and C) following the injection of different doses of estradiol benzoate (data from Gardner and Argyris¹²).

responses to ranges of doses of estradiol benzoate used are presented also in FIGURE 6. The vaginal tissues from mice of the A strain required injections of 0.125 $\mu\text{g.}$ of estradiol benzoate for a 50 per cent $\pm$ 10 per cent response (26 of 45 tests), whereas vaginal tissues of C_{57} -strain donors required 0.0625 $\mu\text{g.}$ of estradiol benzoate (28 of 50 tests). Only 75 per cent of the transplanted A-strain vaginas responded after injection of 0.25 $\mu\text{g.}$ of estradiol benzoate and 90 per cent of the C_{57} -strain vaginas. The transplanted tissues from both of the parental strains required about twice as much estrogen for a response compa-

rable to that induced in the intact vaginas. The same order of strain differences, however, was maintained. These observations led to the conclusion that the strain differences are intrinsic to the vaginal tissue, and not to strain differences in the metabolism, inactivation of estrogen, antagonizing hormones, or humoral influences.

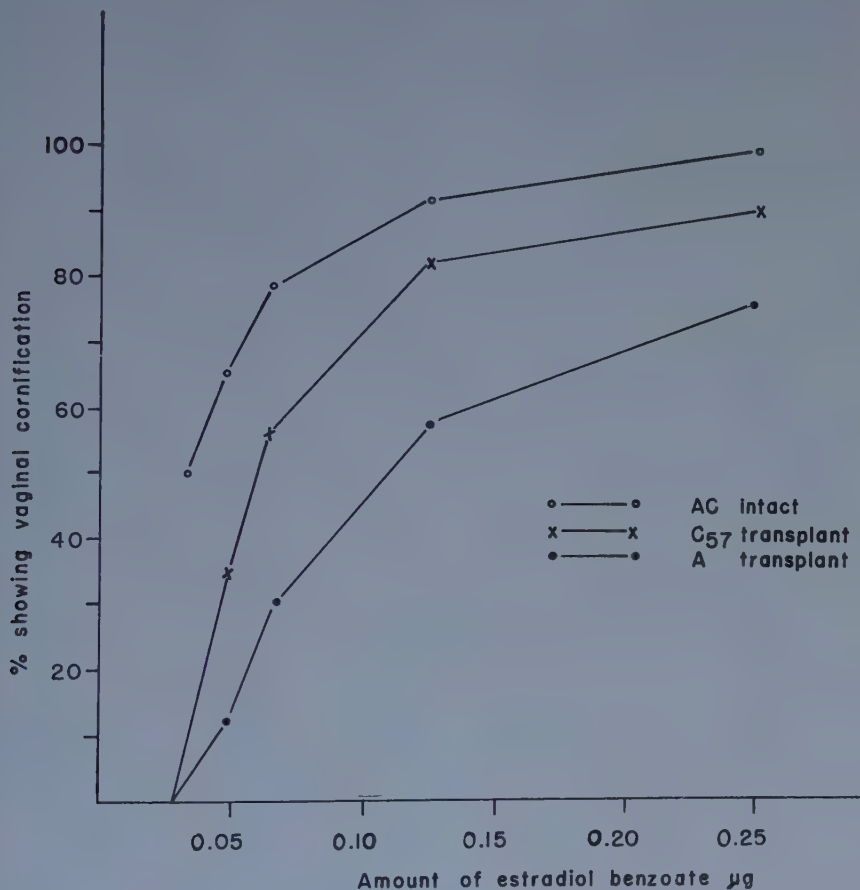


FIGURE 6. Response of transplants of vaginas of mice of the A and C₅₇ strains in hybrid hosts and of the hosts' own vaginas to different amounts of estradiol benzoate (data from Gardner and Argyris¹²).

Quantitative differences in the sensitivity of the mammary glands also were demonstrated.^{10, 15} These differences were determined in part by early exposure to hormones.¹⁵ The possible influences of a usual hormonal environment during early life on the subsequent responsiveness of the vaginal mucosa to estrogens was investigated. Mice of three hybrid groups (C₅₇ × CBA — CC, A × C₃H — AC, and A × C₅₇ — A₇) were used. Testosterone propionate, 0.50 mg. dissolved in 0.02 cc. of sesame oil, was injected subcutaneously when the mice were approximately 2, 9, and 16 days old. The mice were weaned

when approximately 25 days old and ovariectomized when 17 to 21 days old. Subsequently, single subcutaneous injections of amounts of estradiol benzoate ranging from 0.0398 to 1.25 $\mu\text{g.}$ were made sequentially in order to test minimal vaginal responsiveness to the estrogen and the duration of effect (TABLE 1). The duration of vaginal cornification was prolonged when the larger doses of estrogen were given in both groups that received testosterone and in their controls (FIGURE 7). The periods tended to be longer among the androgen-treated mice, however. Some periods of vaginal cornification among these

TABLE 1

VAGINAL CORNIFICATION AFTER INJECTION OF DIFFERENT AMOUNTS OF ESTRADIOL BENZOATE INTO OVARECTOMIZED MICE THAT HAD RECEIVED TESTOSTERONE PROPIONATE IN EARLY LIFE AND HAD SERVED AS CONTROLS

	Group and treatment					
	AC(TP)*	AC-C*	A ₇ (TP)	A ₇ -C	CC(TP)	CC-C
No. of mice receiving:	5	3	21	13	21	13
1.25 $\mu\text{g.}$						
No. of tests/No. ++*	1/1	2/2	6/6	3/3	7/7	11/11
Average days ++	8	7	13	9	13	8
0.625 $\mu\text{g.}$						
No. of tests/No. ++	4/4	3/3	6/6	13/13	3/3	13/13
Average days ++	7	4	8	5	6	6.6
0.3125 $\mu\text{g.}$						
No. of tests/No. ++	4/4	3/3	4/4	13/13	4/1	17/17
Average days ++	5	5	6	4	11	5.6
0.156 $\mu\text{g.}$						
No. of tests/No. ++	2/2	3/3	4/4	13/13	1/1	9/9
Average days ++	3	2	5	3.5	8	4
0.078 $\mu\text{g.}$						
No. of tests/No. ++	3/3	3/3	4/4	16/14	2/2	13/13
Average days ++	1	2	4	2	4	3
0.0398 $\mu\text{g.}$						
No. of tests/No. ++	5/3	7/2	6/3	22/16	2/2	24/19
Average days ++	0.6	0.3	0.5	1.2	2	2

* Key: TP, 3 injections of testosterone propionate were injected at weekly intervals beginning at 2 or 3 days of age; C, mice that received no androgen. ++ refers to the estrous, proestrous, or early postestrous type of vaginal smear.

mice after they attained ages of 50 to 150 days continued more or less indefinitely or for prolonged periods (FIGURE 8).

Most of the testosterone-propionate-treated mice showed spontaneous vaginal cornification for prolonged periods, that is, vaginal cornification occurred in the absence of extrinsic estrogen or of obvious sources of intrinsic estrogens and in the presence of relatively poorly developed uteri. Eight of the A₇ mice began to show prolonged periods of vaginal cornification during the second 50-day period and over half of the mice showed continued vaginal cornification after 100 days of age (FIGURE 8). The consistency of the observations within each group and between groups was striking. Prolonged periods of vaginal cornification occurred only in mice one year of age, or older if testosterone had not been given early in life, and then only in two mice that showed adrenal cortical hyperplasia.

Critical data were not obtained for the minimal effective dose at the 50 per cent $\pm$ 10 per cent level. The indications are that threshold amounts were not changed, insofar as this could be determined. Only a limited number of tests could be conducted with the androgen-treated mice because they began

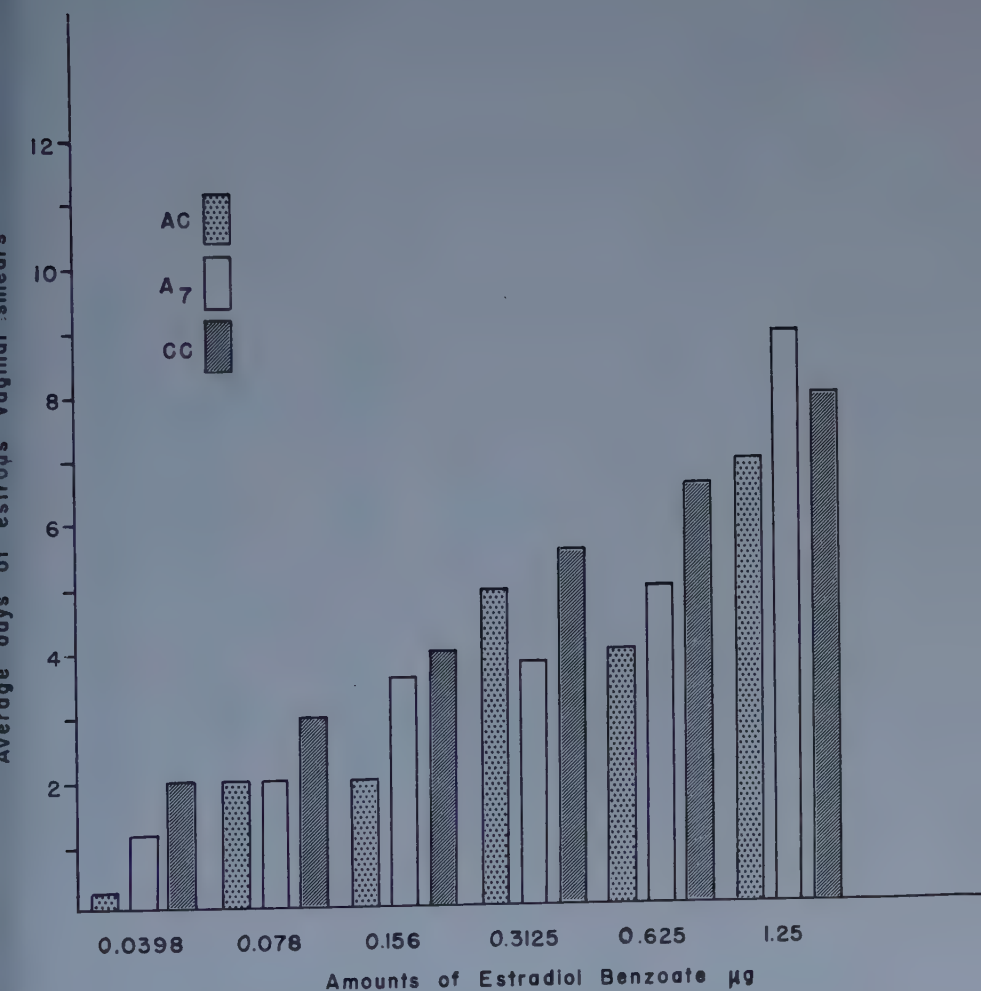


FIGURE 7. Average number of days of vaginal cornification that were noted in 3 groups of hybrid ovariectomized mice following the subcutaneous injection of different amounts of estradiol benzoate.

to show prolonged estrous vaginal smears without any injected extrinsic hormone (TABLE 1). Nine of the A₇ mice began to show continuous vaginal estrus before any extrinsic estrogens were injected.

At this time I must interpret the data obtained from the experiments described above as indicating that testosterone propionate in some way modifies the vaginas of ovariectomized mice to cause them to exfoliate cornified cells

without added extrinsic estrogens. Obvious internal sources of estrogen were lacking. The diet consisted of Purina Lab Chow. Male mice fed this diet concurrently failed to show mammary growth: a sensitive test for dietary estrogens. Techniques are available to elucidate this problem further. It does seem at this time that exposure to a hormone at an early age may permanently modify responsiveness of the vagina.

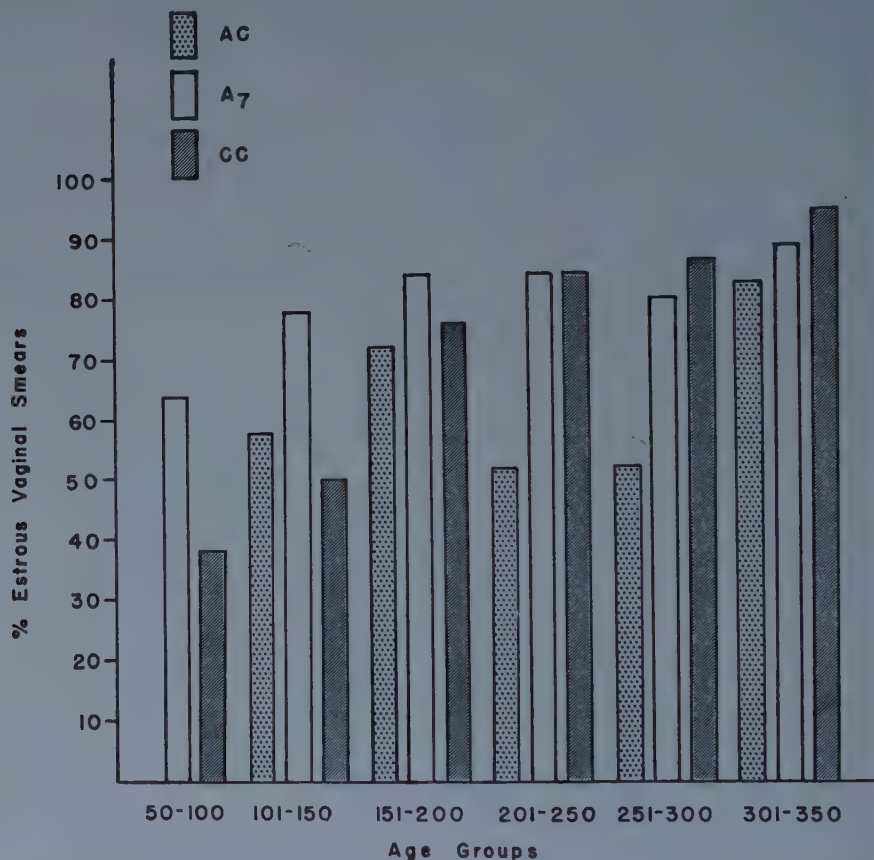


FIGURE 8. Average per cent of days of successive 50-day periods during which ovariectomized hybrid mice of 3 groups that had received testosterone propionate during the first 3 weeks of life showed cornified vaginal smears without injection of extrinsic estrogens.

The earlier experiments in my laboratory convincingly indicated that strain differences in the responsiveness of the vaginal mucosa to estrogens were in the vaginal tissue itself. This may be determined, not by a direct action of the genotype, but by the ontogenic experiences of the developing vagina.

Discussion

Different investigators in different laboratories have observed considerable strain differences in the spontaneous estrous cycles and in the vaginal responses

to injected estrogens (TABLES 2, 3; FIGURES 9 to 11). Considerable data are available and are reported here to indicate that the vagina itself contains the mechanism whereby these differences exist. Under such circumstances it would seem illogical to do elaborate assays of intrinsic steroids to account for individual differences in vaginal responsiveness. Each end organ must be considered as an entity with some predetermined capacity to react to its environment or to specific thresholds of a variable environment.

TABLE 2

CHARACTERISTICS OF THE ESTROUS CYCLES OF MICE OF SEVERAL DIFFERENT INBRED STRAINS AS DETERMINED BY EXAMINATION OF SMEARS OF EXFOLIATED CELLS*

Strain of mice	On diet 1 to 32 days				On diet 2 to 28 days			
	Days of keratinized smears		Days of keratinized smears/cycle	Number of cycles	Days of keratinized cells		Average number of cycles	Days keratinized/days diestrous
	Mean	Range			Mean	Range		
NB	16	9-20	3.0	5.6	17	13-20	4.6	1.57
A	15	10-20	2.7	5.7	20	18-22	3.8	2.50
OB	15	2-21	3.2	4.6	14	8-18	3.6	1.03
C ₅₇	13	6-23	2.9	4.6	9	4-17	3.6	0.50
CBA	12	8-17	1.8	6.7	14	13-15	5.0	1.00
D	11	7-15	2.4	4.6	6	5-9	3.1	0.30
M	10	2-13	1.8	5.5				
C ₃ H	9	5-12	1.6	5.6				
Y	7	4-14	1.8	3.7				
N	6	4-8	1.1	5.5				

* Data from Burns *et al.*⁴ and Suntzeff *et al.*⁵

TABLE 3

RELATIVE SENSITIVITY OF THE VAGINAS AND MAMMARY GLANDS OF MICE OF DIFFERENT STRAINS TO ESTRONE INJECTED SUBCUTANEOUSLY IN OILY SOLUTION*

Strain	Relative vaginal responsiveness	Relative mammary responsiveness
C ₅₇	1	1
O ₂₀	1	2
Riet	1	10
dba	7	1

* Data from Mühlbock.¹⁰

The vaginal response determined here was a qualitative one: the appearance of a large number of cornified exfoliated cells. This response depends upon a cellular change of both nuclear and cytoplasmic substances and possibly also upon a preceding proliferation of the basal cells to increase the layers of the stratified squamous epithelium. At this time it is not known whether proliferation is the essential qualitative entity controlled by the hormonal environment, whether it is cornification, or whether the two are inseparable. The limited studies to date have indicated that vaginal cornification occurred only when the stratified epithelium had become thickened by proliferation, whether in the intact vagina or in vaginal transplants.

Proliferation of cells of the basal layer of the vaginal epithelium begins within a few hours after the injection of estrogens into ovariectomized mice.^{16, 17} The surface layers, preexisting at the time stimulation was begun, were sloughed without undergoing cornification. Only the recently formed cells, under suitable stimulation, seemed to undergo cornification as if the two qualitative responses were determined simultaneously.

Conditions that may affect certain specific metabolic processes or the general nutritional level of the animal also may influence quantitative or qualitative aspects of the vaginal response to estrogens. The rate of keratinization of

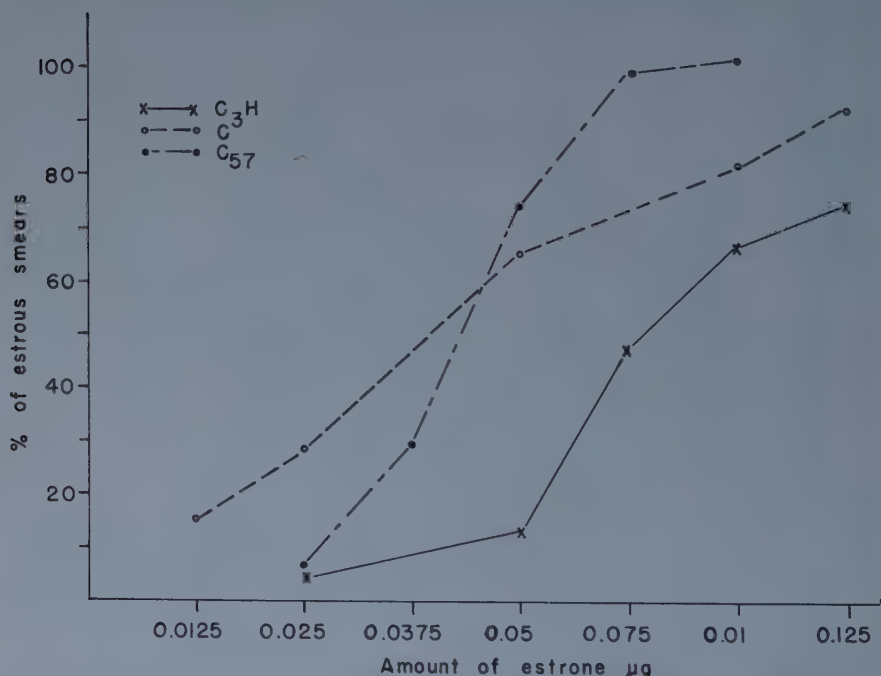


FIGURE 9. Strain differences in sensitivity to subcutaneous injections of estrone (re-tabulated from data of Shimkin and Andervont⁷).

vaginal epithelium was stimulated by insulin,¹⁸ which was assumed to stimulate phosphorylation and transport of glucose into the cells. The intravaginal application of insulin in amounts too small to produce systemic effects augmented vaginal cornification and glycogen storage, and did not appear to depress mitotic activity; phloridzin inhibited keratinization. Hypo- and hyperthyroidism also modified the response of the vagina to estrone.¹⁹

Vitamin A also inhibits vaginal cornification, and deficiencies thereof augment vaginal cornification.²⁰ Likewise, the intravaginal application of vitamin A prevented cornification of the vagina in response to the topical application of estrogen,²¹ and without modifying the growth of the epithelium as estimated by thickness. Vitamin A by itself had no appreciable vaginal growth-stimulating effect, and seemed to influence primarily the tendency for the cells to

keratinize. Although hepatic storage of vitamin A tended to be higher in mice of the A strain than in mice of the C₅₇ strain, the differences were of no statistical significance.²² Studies should be done, however, on the vitamin-A levels of the vaginal tissue itself.

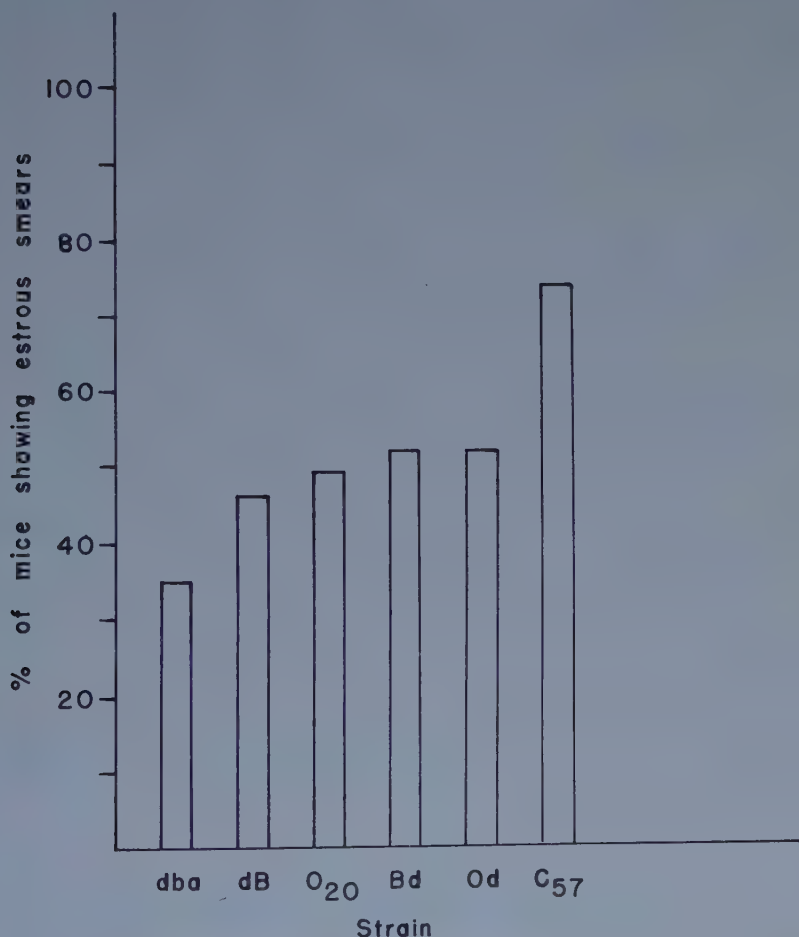


FIGURE 10. Per cent of mice from 3 different inbred strains (dba, O₂₀, C₅₇) and 3 hybrid groups showing estrous responses to comparable doses of estrone (data from van Gulik and Korteweg⁸).

Summary

Earlier publications were reviewed which reported that strain differences exist in the regularity and length of estrous cycles and in the duration of estrus in mice of different inbred strains.

Strain differences existed in the sensitivity of vaginal cornification response of ovariectomized mice to injected estrogens or to the topical application of estrogen.

Vaginas of mice from strains with different sensitivities to estrogen, when transplanted subcutaneously in their hybrid offspring, retained different sensitivities to estrogen when in the same host. The difference in sensitivity of the vagina to estrogen was in the vaginal tissue itself and had been determined, apparently, by the sixteenth day, when some of the grafts were made.

The injection of testosterone propionate into mice during the first two to three weeks of life resulted in little immediate change in vaginal sensitivity to

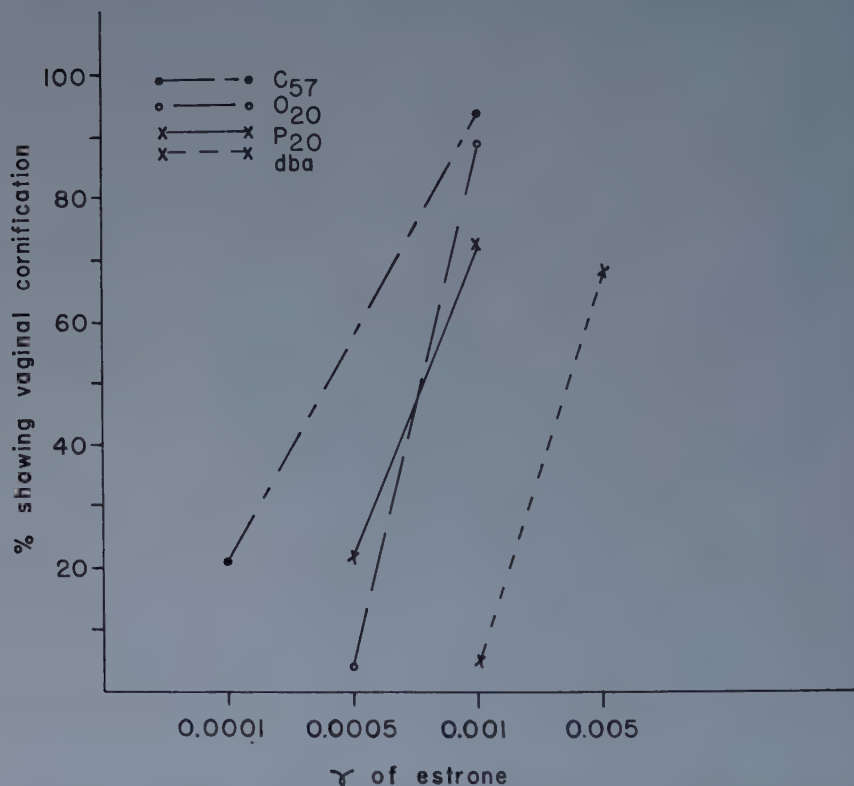


FIGURE 11. Strain differences in vaginal estrous responses to estrone (2 applications within 24 hours) applied intravaginally in 50 per cent glycerol (data from Mühlbock^{9, 10}).

estrogen, but in a consistent and early appearance of prolonged vaginal cornification in the mice after castration at 17 to 21 days of age.

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THE MODIFICATION OF ESTROGEN-INDUCED CHANGES IN RAT VAGINAS WITH STEROIDS AND RELATED AGENTS

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The interactions of steroid hormones have interested endocrinologists for more than 30 years. However, it is only recently that workers in the field regularly have begun to suggest that these interactions might perform essential functions in the control of sexual development and cyclic sexual phenomena. In fact, many workers seem to have considered interactions more as pharmacological curiosities than as important physiological mechanisms by which cyclic and developmental changes may be produced. Although it may be premature to take a dogmatic position, I feel that the interactions of steroid hormones deserve serious consideration and will be found to contribute greatly to our knowledge of the physiology of sex.

In attempting to sort out these relationships, substances that interact with estrogens have been separated into several categories based upon the most obvious biological activity of the material in question. Although the borders are indistinct and there is considerable overlap among groups, these compounds are designated as estrogens, androgens, progestins, and gluco- and mineralocorticoids. One compound from each of these categories has been selected as an example and studied in combination with estrogens. This "type" approach must be interpreted with caution, since slight structural modification can often result in remarkable changes in the pharmacological properties of a compound. However, it is my opinion that these data can be generalized to give us an idea of the way in which compounds within certain broad categories will interact with estrogens.

The difficulty of relating the results obtained in one laboratory to those obtained in another is one of the major blocks to generalization of the mass of accumulated data on interactions. Species differences and, where species are the same, variations in protocol and strain of animal result in major differences of interpretation and subsequent philosophy. I have attempted to study examples of the classes of compounds mentioned above, employing a single protocol for all materials; in this way valid comparisons among the compounds were sought within the limits of a single assay procedure. Occasionally, the glowing hope that a modified approach would clarify a given problem has resulted in procedural changes; such experiments generally have added to our already more than adequate confusion.

This paper summarizes studies concerning the interactions of steroid hormones on the vaginas of spayed rats. In addition, the same series of compounds has been under examination in two types of uterine growth assays. These experiments, which were carried out in association with my colleagues D. W. Calhoun and R. L. Elton, are the subjects of several previous papers and some that remain to be completed. Certain of these other experiments will be noted briefly.

Materials and Methods

Almost all of our studies have employed a colony of rats prepared according to the procedures described previously.^{1, 2} Briefly, Sprague-Dawley rats were spayed at 60 days of age and then allowed 30 days to recover from the effects of the operation. After recovery, the rats were primed with 2.5 μ g. of estrone at 0 and again at 24 hours; each priming dose was contained in 0.1 ml. of corn oil that was administered subcutaneously. Vaginal smears were taken at 56 and 72 hours. New rats were integrated into the colony only after they had responded positively to 2 courses of priming. Assay colony rats were primed in the same way on alternate weeks; only those that had responded positively to priming the previous week, generally about 90 per cent, were employed for testing. Most of our studies were based upon fresh smears that were scored according to the method of Biggers and Claringbold,³ in which the absence of leukocytes is considered a positive response to a test material and all other smears are considered as negative. Test substances were generally handled exactly as noted above for priming. When two materials were administered simultaneously, they were mixed together and injected in a single volume of corn oil. Within this generalized protocol 3 experimental designs were employed. First, a modification of Horn's ED₅₀ method was used:⁴ 4 dose levels of the standard estrogen, chosen at equal log distances, were administered both alone and in combination with another steroid. Effect was evident from changes in estimated ED₅₀ values. Second, in some experiments, an arbitrary dose of estrogen was administered to a large number of rats; subgroups of these also received varying doses of the test material. Third, in our studies on the interactions of estrogens¹ we attempted to work exclusively at the ED₅₀ dose level. When we tested pairs of compounds, two groups received the estimated ED₅₀ of each substance; a third group, usually with twice as many rats as each of the other groups, was injected with one half of the ED₅₀ dose of each material. Unless specified in the text, all experiments to be summarized were of these types.

In some experiments each group of rats received at 0 hours a single injection that contained estrogen either alone or mixed with test steroid; smears were taken at 24, 48, 72, and 96 hours. These smears were stained with Shorr's single differential stain,⁵ and evaluated at the same time. The microscopic examinations were done in a quasi-blind manner; the reader did not know which animals had received which treatment. However, the experimental design was known, as was the order of the animals.

Throughout this paper the doses indicated are total doses, whether administered in one, two, or more injections.

Interactions of Estrogens on the Vagina

Hisaw *et al.*⁶ were the first workers to point out that estriol and estrone both inhibited the action of estradiol-17 β on the uteri of spayed rats and that estriol depressed the uterine response to estrone. Huggins and Jensen,⁷ who employed hypophysectomized rats, confirmed the estrone-estriol interaction and implicated a series of other phenolic steroids as estrogen antagonists. Huggins and

Jensen also examined the vaginas of their rats, but found no remarkable evidence of interaction.

We (Edgren and Calhoun¹) concentrated on the vaginal smears of rats and, since our preliminary data had suggested a simple additive response, we re-

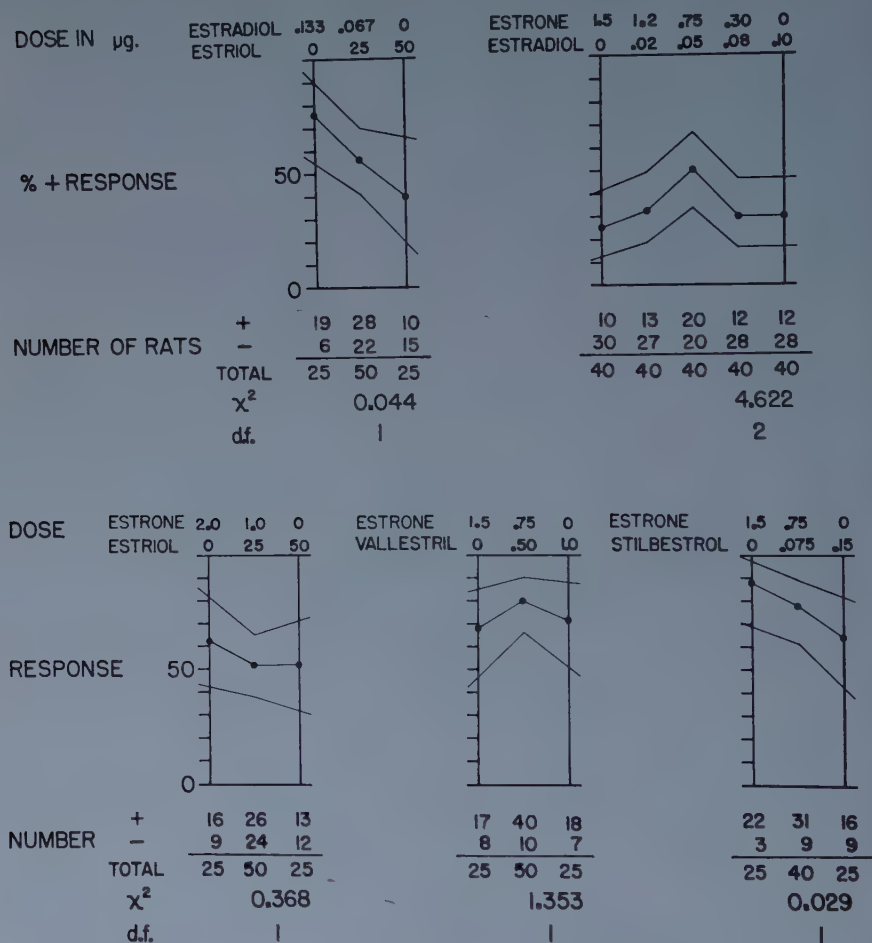


FIGURE 1. Effects of estrogen combinations on vaginal cornification in spayed rats. Observed percentages of positive responses are graphed at each dose or combination. Percentages are bracketed by their 95 per cent confidence limits. The object of the experiment was to determine whether any of the intermediate mixed doses produced a nonintermediate response. The Chi-square tests were employed for agreement with a linear interpolation of the mixture point (or points) between the values for the two pure materials. No pair of estrogens produced significant curvature. Reproduced by permission from *The American Journal of Physiology*.¹

stricted our study to the ED_{50} level. In these experiments we found that one half of the ED_{50} dose of one estrogen could substitute for one half of the ED_{50} dose of another without significant change of biological activity (FIGURE 1). The following pairs of compounds were tested: estrone and estradiol-17 β ; es-

trone and estriol; estrone and diethylstilbestrol; estrone and Vallestril*; and estradiol-17 β and estriol. In each case the less potent material acted simply as a dilution of the other; thus the interactions were additive in the simplest sense of the word.

Since publication of these data we have been disturbed by the apparent discrepancy between the uterine response to estrone-estriol mixtures and the response of the vagina to similar mixtures. Recently we completed a preliminary experiment in which we studied both uteri and vaginas of rats treated with estrone, estriol, or mixtures of the two. In an earlier study⁸ we had found that the metrotropic effects of estrone were inhibited by relatively low doses of estriol, but only over a narrow range of doses of estrone (see also Huggins and Jensen⁷). Below this range the estriol appeared to swamp the estrone, since the response could not be distinguished from that produced by the estriol alone; above this range the addition of estriol had no apparent effect upon the near-maximal response to the estrone. A portion of this experiment was repeated, selecting a dose of estrone below the inhibitory range (0.3 μ g.), one well within it (3.0 μ g.), and a third above it (30.0 μ g.). The rats used had been spayed at 30 days of age and were then allowed about 10 days to recover from the effects of the operation. The materials were administered in 3 equal doses over a 3-day period, and the rats were sacrificed 24 hours after the final injection. At autopsy the uteri were removed, cleaned of adherent tissue, scored and blotted to remove excess fluid, and weighed wet on a torsion balance. Immediately before autopsy the vagina was examined for rupture of the vaginal membrane; if the plate was open, smears were taken. In this study the expected inhibition of estrone-induced uterine weight by estriol was obtained; however, of the 4 indices of action on the vagina (membrane opening, smear, epithelial height, and formation of a keratinized sheath lining the lumen) none showed any marked effect of added estriol (TABLE 1). Since there was certainly no inhibition of the vaginal response, it would appear that estrone-estriol interactions are markedly different in the two organs. An experiment is now in progress employing the protocol that produced apparent addition of materials with respect to the vaginal smear; both the uteri and vaginas of such rats will be examined.

This experiment localizes the point of interaction of estrone and estriol in the target organ. Prior studies from this laboratory have not eliminated other potential sites of interaction, since they did not evaluate effects on the two targets simultaneously. For example, the data of Szego⁹ strongly suggest that estradiol and cortisol compete for a protein transport vehicle in the blood, rather than for some site in the target organ itself. In our experiment, if interaction had been for some blood protein, the two targets would have shown similar patterns of responses. The differences demonstrated by the uteri and vaginas restrict the interaction to the level of the target organ.

Effect of Androgens on the Vaginal Response to Estrogens

For almost 25 years androgens have been known to antagonize the effects of estrogens. In 1935 de Jongh, in two papers, and Korenchevsky and Den-

* Product of G. D. Searle & Co., Chicago, Ill.

nison (Emmens and Parkes¹⁰) showed that androgens would protect male sex accessories from the pathogenic effects of estrogens. Gardner and Pfeiffer¹¹ noted that the effects of ovarian grafts on bone were greater in castrated than in intact males. These authors showed that they could block the proliferative effects of estradiol benzoate on medullary bone with simultaneous testosterone propionate (TP). Robson, in 1936, demonstrated that testosterone inhibited the estrous cycles of intact and the vaginal response of ovariectomized mice to estrone.¹² In 1938 he¹³ compared the effects of various androgens upon the vaginal changes induced by estrone or estradiol; later¹⁴ he concluded that these interactions were not at the level of the vaginal cells, since the blockers he employed were not markedly more potent when given intravaginally. This is in contradistinction to the more recent findings of Müller.¹⁵ Robson and

TABLE 1
EFFECT OF ESTRIOL UPON THE RESPONSE OF THE UTERI AND VAGINAS OF
SPAYED FEMALE RATS TO ESTRONE

Dose (μg.)		N	Uterus wt. (mg.)	Vaginal response			
Estrone	Estriol			+ Smears	Membrane opening	Cell height (No. cells)	Keratiniza- tion
0.3	0	21	34.8	13/15 17/17	1/21	3-4	0
0.3	30	22	66.1		0/22	6	0
3.0	0	21	95.2		0/21	10	+
3.0	30	21	75.3		1/21	10	+
30.0	0	21	138.3		15/21	10	+++
30.0	30	22	124.1		17/22	10	+++
0	0	20	32.8		0/20	3-4	0
0	30	19	60.9		0/19	6	+

Doses indicated were administered in 3 injections, 24 hours apart; the rats were sacrificed 24 hours after the third injection. Keratinization of +++ indicates the presence of a keratinized sheath lining the lumen; + indicates that epithelial cells were flattened toward the lumen, but that no sheath was present; N represents the number of animals tested at each dose.

* Bracketed groups differ significantly with $p < 0.01$.

† Bracketed groups differ significantly with $p < 0.05$.

Sharaf¹⁶ showed that methyl testosterone was a strong inhibitor of estradiol-induced vaginal changes, but was only poorly effective against changes produced by synthetic estrogens. TP was a poor blocker of estradiol stimulation and had no effect against stilbestrol, doisylnolic acid, allenolic acid, or triphenyl-iodoethylene.

Arhelger and Huseby¹⁷ transplanted vaginal sections into male mice, and found that endogenous testoids markedly antagonized the effects of estrone and estradiol. Takewaki¹⁸⁻²¹ has also analyzed the effects of estrogens on vaginal transplants. He was able to show that a subcutaneous graft was atrophic, and responded to estradiol in a manner similar to that of the vagina of spayed females; on the other hand, 50 to 100 times as much estradiol was needed to stimulate a similar response in the intratesticular transplants, which were greatly mucified in control animals.¹⁸ Mucification of subcutaneous grafts was produced also with TP. Experimental cryptorchism increased the

estradiol sensitivity of intratesticular grafts approximately fivefold, whereas unilateral castration decreased graft sensitivity to exogenous estradiol.¹⁹ When both ovary and a vaginal section were transplanted in juxtaposition, the subcutaneous vaginas were cornified; the intratesticular grafts were mucified.²⁰ Cryptorchidectomy of these graft-bearing testes allowed cornification to occur at low doses of estradiol.²¹ These four papers show that endogenous androgens inhibit the effects of estrogens on the vagina, and that conditions that would be expected to reduce the secretion of testoids increase the vaginal sensitivity to the estrogens.

TABLE 2

EFFECTS OF TESTOSTERONE PROPIONATE AND 17-ETHYL-19-NORTESTOSTERONE ON ESTRONE-INDUCED VAGINAL CORNIFICATION IN SPAYED RATS

Dose (μ g.)		N	Percentages of + response
Estrone	Testosterone propionate		
1.5	0	20	80
1.5	50	20	30*
1.5	100	20	25*
1.5	250	16	31*
1.5	500	20	50*
1.5	1000	20	31*
	17-ethyl-19-nortestosterone		
1.5	0	40	70
1.5	25	24	67
1.5	50	24	58
1.5	100	24	75
1.5	250	20	32*
1.5	500	28	18*
1.5	1000	28	11
1.5	2000	28	4
1.5	4000	12	25
1.5	8000	12	33

Materials were administered in 2 injections separated by 24 hours. The data on 17-ethyl-19-nortestosterone were pooled from 4 separate experiments. Data from Edgren.²

* Significantly different from estrone group with $p < 0.01$.

Recently² the effects of TP and 17-ethyl-19-nortestosterone on estrone-induced changes in vaginal cytology were discussed. When increasing doses of either TP or 17-ethyl-19-nortestosterone were given simultaneously with a dose of estrone that produced a positive response in about 75 per cent of the rats, 50 μ g. of TP and about 250 μ g. of 17-ethyl-19-nortestosterone produced significant decreases in response (TABLE 2).

Both TP and 17-ethyl-19-nortestosterone at single doses of 1 mg. partially blocked the vaginal changes produced by one 10- μ g. injection of estrone. The 0-hour smears from all groups showed the typical castrate condition, which was not altered in the controls or in groups receiving testosterone or 17-ethyl-19-nortestosterone. The group that received estrone alone showed complete

keratinization of the vaginal epithelium by 48 hours and a return to castrate condition by 96 hours. When either testoid was administered with the estrogen, keratinization appeared to proceed in a relatively normal manner, but leukocytes were never absent from the smear.

A third experiment studied the time at which these testoid actions are effected. One mg. of 17-ethyl-19-nortestosterone was administered at -24, 0, or +24 hours, and 10 μ g. of estrone, at 0 hours. Pretreatment and simultaneous treatment of estrone-treated rats with 17-ethyl-19-nortestosterone resulted in smears containing leukocytes and keratinized epithelial cells; how-

TABLE 3

EFFECTS OF TESTOSTERONE PROPIONATE (TP) ON THE VAGINAL RESPONSE TO ESTRONE

Dose (μ g.)		N	+ Response	Percentages of + response	ED ₅₀ (μ g.)
Estrone	TP				
1	0	115	24	21	2.1
2	0	115	53	46	
4	0	115	90	77	
8	0	115	103	90	
2	250	37	3	8	4.1
4	250	37	22	60	
8	250	37	29	78	
16	250	37	33	89	
4	500	38	4	10	7.7
8	500	38	25	66	
16	500	38	30	79	
32	500	38	35	92	
8	750	38	9	23	19.6
16	750	38	19	50	
32	750	38	22	58	
64	750	38	33	87	
8	1000	36	8	23	17.4
16	1000	36	15	42	
32	1000	36	28	78	
64	1000	36	33	90	

Materials administered in 2 injections separated by 24 hours. ED₅₀ by method of Horn.⁴

ever, leukocytes were absent from smears of animals that received androgen posttreatment. Thus, by 24 hours after estrogen administration the changes leading to vaginal estrus had progressed to a point at which androgen blockade was no longer possible.

Attempts to quantify these relationships have not been entirely satisfactory, although analyses have been completed of the effects of varying doses of testosterone propionate on the ED₅₀ of estrone in the standard assay. The experimental design and results are summarized in TABLE 3. The greater the amount of testosterone, the larger the amount of estrone required to produce a 50 per cent response in treated animals, as judged⁴ by the estimated ED₅₀. The relationship between the estrone ED₅₀ and the dose of testosterone does

not appear to be linear (FIGURE 2). The concavity of the curve would mean that large doses of testosterone are relatively more effective than small doses in inhibiting the vaginal response to estrone. Unfortunately, this reliance on simple ED_{50} may be misleading. Horn's method assumes that the dose-response curve be linear over the range of doses employed; this does not appear to be entirely true for these data (FIGURE 3). The estrone curve is satisfactorily linear, as are the curves for combinations of estrone with TP at 750 μ g. and 1000 μ g. The combinations of estrone with TP at 250 μ g. and 500 μ g. certainly show curvature, which makes the estimated ED_{50} values suspect.

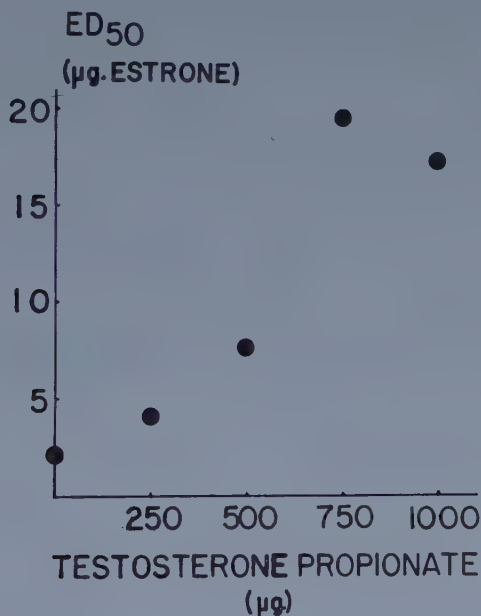


FIGURE 2. The relationship of dose of testosterone propionate to the ED_{50} of estrone administered simultaneously.

These data also have been investigated, employing the isoresponse technique that was of value in understanding the interactions of steroids in a mouse uterine growth assay.²² In this approach the response produced by a given combination of estrone and TP (for example, 500 μ g. TP and 8 μ g. estrone, response 66 per cent) is associated with the amount of estrone alone that would produce an equivalent response (3.5 μ g., data from FIGURE 3). The associated points (8, 500) and (3.5, 0) with arithmetic dosage scales for TP and estrone are plotted on a graph and simply connected by a straight line (FIGURE 4). The assumption that all other points on the line represent dose combinations that would yield the same response (66 per cent) was essentially confirmed in mouse-uterine growth studies. Further studies have not yet been carried out to verify the assumption by further selected dose combinations in the case of vaginal smear experiments. However, in a number of cases in the present experiment, points for combinations of doses of estrone and TP fall close to

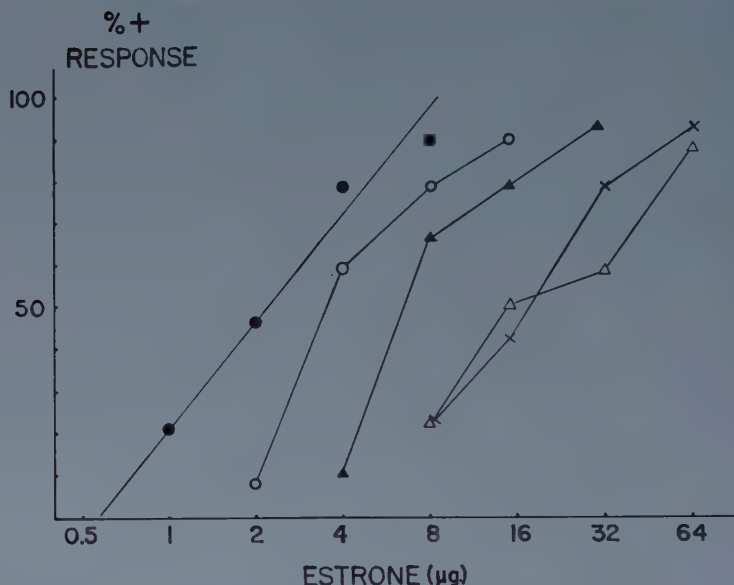


FIGURE 3. The effects of various doses of testosterone propionate (TP) on the dose-response curve for the vaginal smear response. Key: dots, estrone alone; circles, estrone plus 250 μg . of TP; solid triangles, estrone plus 500 μg . of TP; open triangles, estrone plus 750 μg . of TP; x's, estrone plus 1000 μg . of TP.

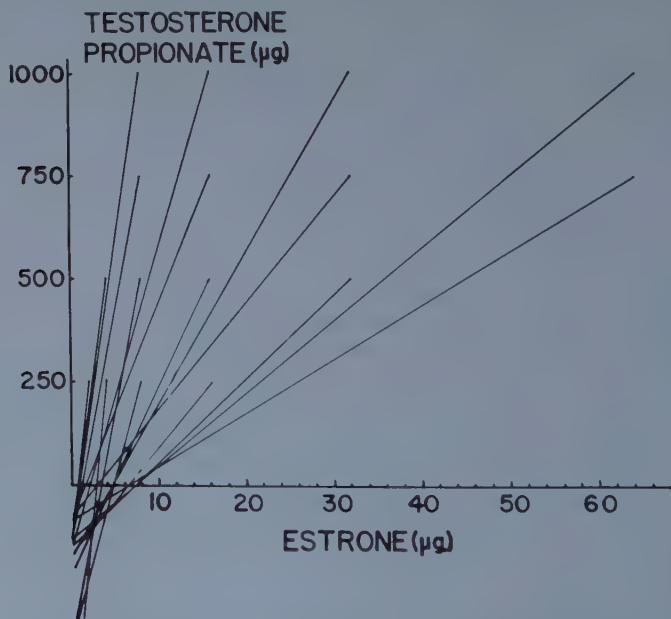


FIGURE 4. Proposed isoresponse lines for the vaginal smear response. Each line connects the point indicating a combination of estrone and TP doses with the point on the abscissa corresponding to the dose of estrone alone that would produce an equivalent response. Scales on ordinate and abscissa are arithmetic.

the lines drawn to other points. For example, the point for the combination of 500 μ g. TP plus 32 μ g. of estrone is very close to the line connecting the 1000- μ g. TP, 64- μ g. estrone point with its pure estrone equivalent, approximately 7 μ g. Thus, estrone alone at 7 μ g. and these two combinations should give approximately equivalent responses if isoresponse lines for vaginal smears are straight. These and a few other such groups of points do, in fact, suggest that these isoresponse lines are straight (TABLE 4).

D. W. Calhoun of our statistical department has determined the form isoresponse lines would be expected to take under a number of simplifying competition assumptions, that is: (1) two hormones, X and Y, react reversibly with a single limited factor such as an activating site for attachment, S; (2) equilibrium is maintained among X, Y, and S, and their complexes XS and YS; and (3) the measured response varies in proportion with the concentra-

TABLE 4

DATA FROM COMBINATIONS OF TESTOSTERONE PROPIONATE AND ESTRONE THAT APPEAR TO SUGGEST THAT ISORESPONSE LINES OF FIGURE 4 ACTUALLY ARE STRAIGHT: THE THREE GROUPS OF DOSAGE COMBINATIONS LIE APPROXIMATELY ALONG STRAIGHT LINES AND SHOULD, BY HYPOTHESIS, PRODUCE EQUAL RESPONSES

Point		N	Percentages of + response
Estrone (μ g.)	TP (μ g.)		
64	1000	36	90
32	500	38	92
6.5	0	—	89
32	1000	36	78
16	500	38	79
4.6	0	—	77
8	1000	36	23
8	750	38	23
4	500	38	10
2	250	37	8
0.7-1	0	—	8-21

tions of S, XS, and YS. Under these circumstances the isoresponse lines are found to be a family of hyperbolas. Each arm of each hyperbola approaches a limiting straight line or asymptote. The curves are so oriented that the asymptotes converge on a single point that will usually be different from the origin (FIGURE 5). Furthermore, it appears that doses of estrone and antagonist used in these experiments were large in the sense that approach of isoresponse lines to the corresponding asymptotic straight-line limits is very close. It is evident from the figure that the vaginal smear data conform reasonably well to the assumptions of competition. These data suggest that we are dealing with a competition between the estrone and testosterone molecules.

A recent experiment examined the effects of a single 1-mg. dose of TP on the vaginal changes produced by 100 μ g. of estriol. In the group that received estriol alone, full-blown cornification occurred at about 72 hours rather than at 48, as was true for estrone. Although most of the smears lacked significant numbers of leukocytes by 48 hours (TABLE 5), they contained appreciable

numbers of unkeratinized epithelial cells. When testoid was added, leukocytes were present in most of the smears at both 48 and 72 hours. In animals that received estriol at 100 μ g., 1000 μ g. of TP produced significant inhibition of vaginal response, whereas lower doses did not have this effect (TABLE 6). These data suggest that the estriol-stimulated vagina is not markedly different from the estrone-stimulated vagina with respect to modification of responsiveness by testosterone.

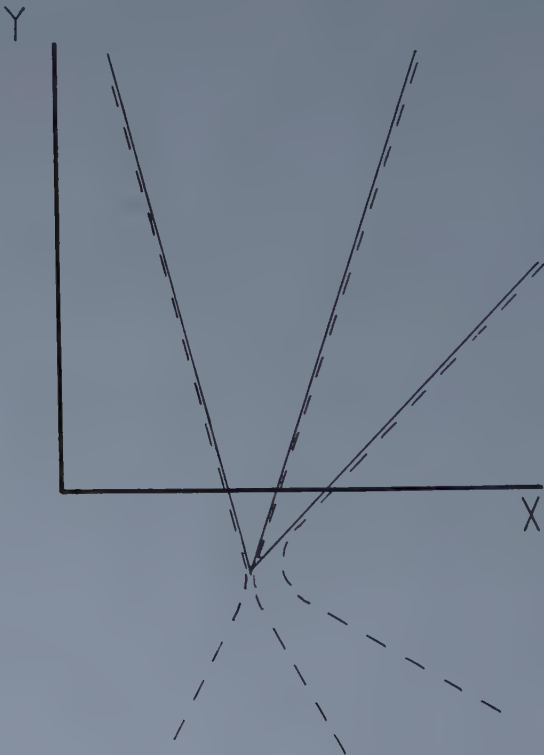


FIGURE 5. Diagram of isoresponse lines based on the assumption that hypothetical hormones *X* and *Y* are in competition. Dosages of *X* and *Y* are on an arithmetic scale. Broken lines represent the hyperbolic isoresponse lines; solid lines, their asymptotes that converge at a point.

TABLE 5
EFFECTS OF TESTOSTERONE PROPIONATE ON THE VAGINAL EFFECTS OF ESTRIOL: ALL MATERIALS ADMINISTERED IN A SINGLE INJECTION AT 0 HOURS

Dose (μ g.)		N	Percentages of + response (hours)				
Estriol	Testosterone propionate		0	24	48	72	96
100	0	10	10	10	80	100	40
100	1000	10	10	30	40	10	10

The demonstration of epithelial keratinization with luminal leukocytes is puzzling. In the normally cycling animal and in spayed rats stimulated to cycle by the administration of estrogen, the loss of vaginal leukocytes occurs together with epithelial keratinization, and these two indices appear to be related causally. The estrogen-stimulated thickening and keratinization of the epithelium may prevent migration of leukocytes from underlying tissues to the vaginal lumen. Consequently, the simultaneous presence of leukocytes and keratinized epithelial elements in the vaginal smear of estrogen-androgen-treated rats might be explained by an incomplete formation of a keratinized vaginal lining in these animals. Whereas it is well known that the epithelial cells of estrogen-treated rats tend to form sheets in the vaginal smears, an incomplete lining might be revealed as a tendency for the cells to occur singly or in small groups in the smears of rats treated with both androgen and estrogen. Re-examination of the slides upon which the earlier paper was based² seemed to support this hypothesis; even FIGURES 4, 5, and 6 of the earlier paper² appeared to show a greater degree of cellular clumping in the estrone

TABLE 6

EFFECTS OF VARYING DOSES OF TESTOSTERONE PROPIONATE ON THE VAGINAL RESPONSE TO ESTRIOL: MATERIALS ADMINISTERED IN 2 INJECTIONS SEPARATED BY 24 HOURS

Dose ($\mu\text{g.}$)		N	+ Responses	Percentages of + response
Estriol	TP			
100	0	12	12	100
100	100	12	9	75
100	300	12	9	75
100	1000	12	7	58*

* Significantly different from controls with $P < 0.05$.

group (FIGURE 6) than in either of the combination groups (FIGURES 4 and 5). In order to gather further support for this contention, 4 groups of 5 rats each were treated as follows: group I, oil; group II, TP, 1000 $\mu\text{g.}$; group III, estrone, 10 $\mu\text{g.}$; and group IV, estrone, 10 $\mu\text{g.}$ plus TP, 1000 $\mu\text{g.}$ Two additional groups were employed: one received progesterone and the other estrone and progesterone; they will be discussed in the next section of this paper. Injections were given at 0 hours; at 48 hours the rats were sacrificed. At autopsy vaginal smears were taken and the vaginas were removed, sectioned at 7 μ , and stained with hematoxylin and eosin for histological study.

The vaginal epithelium of the oil-treated group was essentially atrophic; the epithelium was about 3 cells thick and the outer layer was cuboidal or occasionally low-columnar (FIGURE 6). TP-treated rats showed an atrophic epithelium that was somewhat more compact and had a denser nuclear chromatin pattern (FIGURE 7). Estrone treatment produced the expected proliferation of the epithelium; from the columnar cells of the stratum germinativum the cells became progressively more flattened toward the sheath of flat, keratinized cells that lined the lumen (FIGURE 8). TP, administered with estrone, appeared to block the formation of a sheath of keratinized cells, although the cells lining the lumen were much flattened, and restricted areas of keratiniza-

tion could be found in all animals. Epithelial proliferation apparently had been largely unaffected; however, the nuclei were similar to TP-treated rather than to estrone-treated rats (FIGURE 9). Leukocytes were present in the vaginal smears of all but four of the estrone-treated rats, and were correlated with keratinized cells in the estrogen-androgen group. Thus, smears give a some-

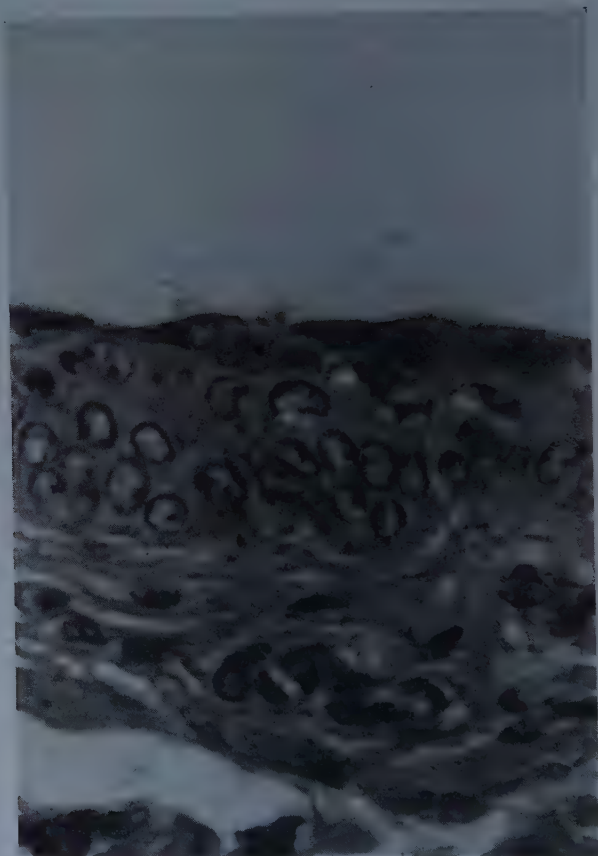


FIGURE 6. Vaginal epithelium of adult spayed rat 48 hours after the administration of corn oil.

what inaccurate picture of the vaginal effects of mixtures of estrogens and androgens.

These data appear to support the hypothesis that testosterone interferes with the formation of a complete, keratinized epithelial sheath in the vagina. Huggins and Jensen⁷ have reported and figured a similar phenomenon resulting from the simultaneous administration of estrone and progesterone.

Effect of Progestins on the Vaginal Response to Estrogens

Progestational agents were the first substances implicated as antagonists of estrogen-stimulated changes in reproductive organs. As early as 1926, Smith

et al. demonstrated mutual antagonisms with respect to vaginal keratinization and pregnancy maintenance.^{23, 24} Robson showed that progesterone blocked vaginal keratinization in estrone- or estradiol-treated, ovariectomized mice,¹³ and concluded from a comparison of systemic administration with intravaginal instillation that the antagonism was not at the level of the vaginal cells.¹⁴ Robson and Sharaf¹⁶ showed that progesterone was a potent antagonist of vaginal

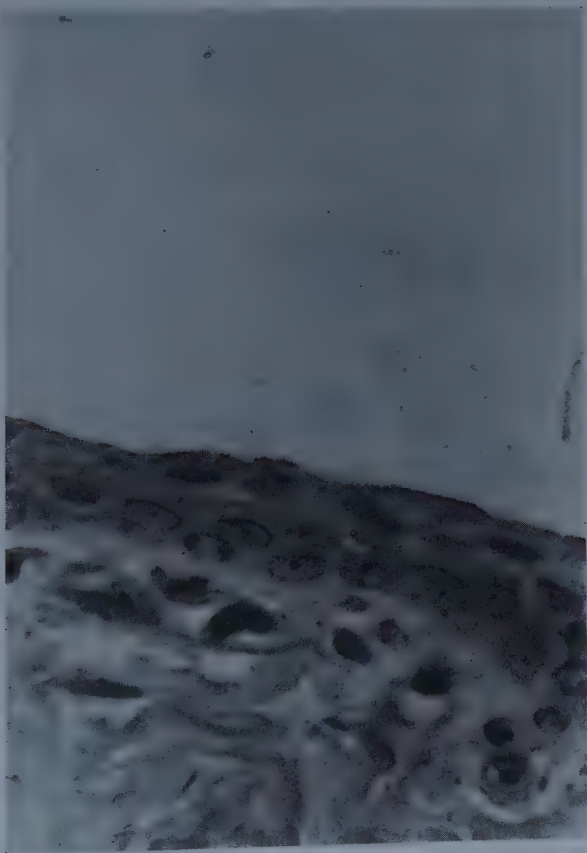


FIGURE 7. Vaginal epithelium of adult spayed rat 48 hours after the administration of 1000 μ g. of testosterone propionate.

changes produced by estradiol, stilbestrol, or doisylnolic acid, but was only weakly effective against allenolic acid or triphenyliodoethylene. Greer²⁵ found that progesterone administration produced a resumption of fairly normal vaginal cycles in persistent-estrus rats that had hypothalamic lesions. Ford²⁶ was unable to demonstrate that progesterone potentiated estradiol-induced vaginal cornification in rats, and inhibition of the estradiol effect occurred only with high doses of progesterone; in contrast, spayed guinea pigs showed changes that were characteristic of natural estrus only when mixtures were employed.²⁷ Three-way mixtures of estradiol, progesterone, and testosterone were found by

Bacher *et al.*²⁸ to produce prolonged estrus when certain dosage combinations were introduced to spayed rats; other dosages did not have such effects. Pretreatment of ewes with progesterone was found by Robinson to reduce the median effective dose of estradiol necessary to produce vaginal cornification from 24 $\mu\text{g.}$ to 14 $\mu\text{g.}$ ^{29, 30}; in addition, the dose-response curve was steeper with the pretreatment, and onset of estrus was earlier. These studies were con-

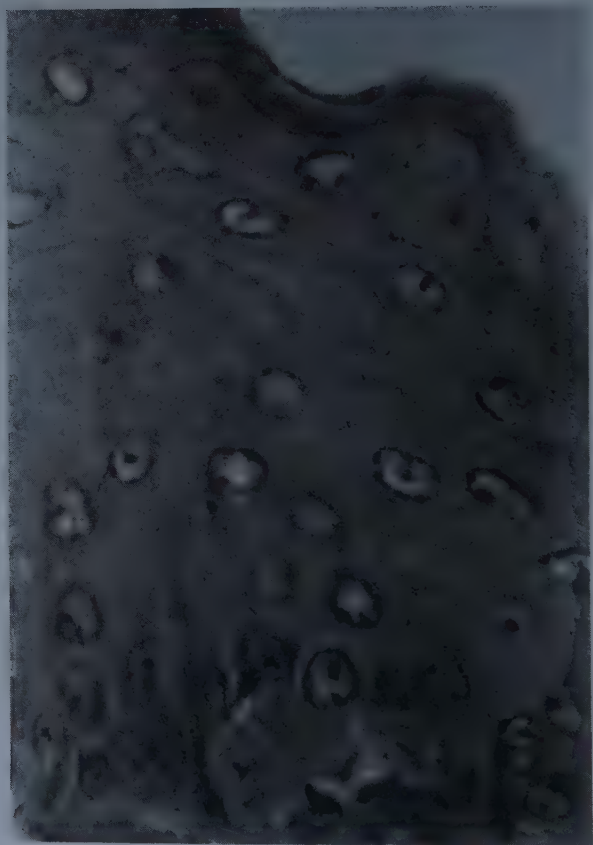


FIGURE 8. Vaginal epithelium of adult spayed rat 48 hours after the administration of 10 $\mu\text{g.}$ of estrone. Note height of epithelium and sheath of keratinized cells lining lumen.

tinued by Moore and Robinson,³¹ who found that estradiol-induced vaginal changes were partially inhibited when estrogen and progestin were given simultaneously; these researchers obtained maximal vaginal responses when estrogen followed progesterone by 48 hours or more. Although time relations differed, estrus behavior, which was evaluated on the basis of proportion of ewes serviced by rams, responded similarly. Perhaps the most remarkable finding was that, as the interval between the final progesterone injection and the initial estradiol injection was increased, there was no effect upon vaginal smear response, but that the proportion of ewes exhibiting estrous behavior de-

creased rapidly. Velardo³² showed that the length of the estrous cycle of progesterone-treated rats was increased over controls, whereas 17 α -hydroxyprogesterone 17-*n*-caproate was essentially ineffective; whether this effect on the cycle resulted from an antagonism of endogenous estrogen or from a pituitary blocking effect of the progestin is unclear.

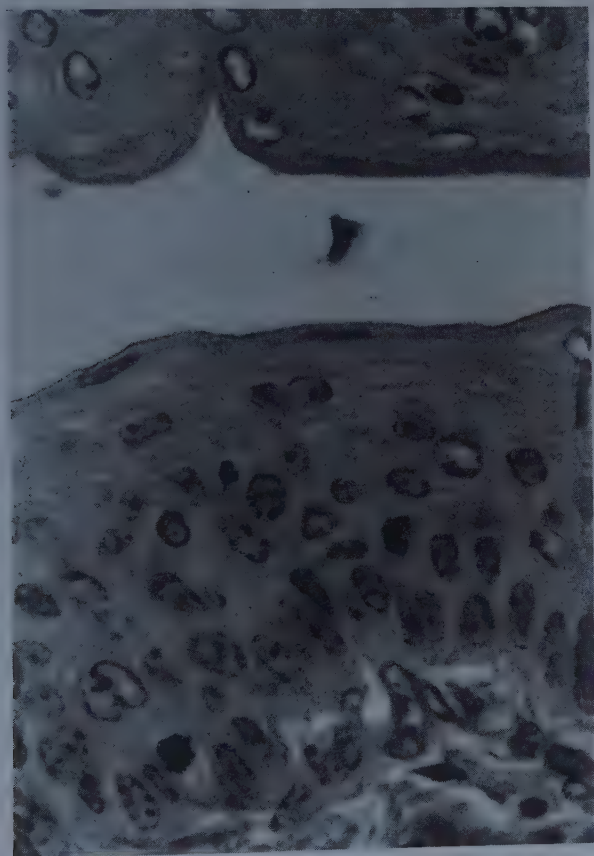


FIGURE 9. Vaginal epithelium of adult spayed rat 48 hours after the administration of 10 μ g. of estrone and 1000 μ g. of testosterone propionate. Note absence of continuous sheath of keratinized cells, height of epithelium, and presence of squamous, partially keratinized plaques.

My experiments with estrogens and progesterone have been somewhat disappointing, since I have been unable to demonstrate any marked effect of progesterone on either estrone- or estriol-induced vaginal changes (TABLES 7 to 9). The data of TABLE 8 may suggest some slight interference with the full-blown cornification expected at the higher dose levels of estrone. In the experiment described in the section on estrogens and testoids, groups of rats also were treated either with 1 mg. of progesterone alone or with a combination of progesterone, 1 mg., and 10 μ g. of estrone. The animals were sacrificed 48 hours

TABLE 7

EFFECTS OF INCREASING AMOUNTS OF PROGESTERONE ON ESTRONE-INDUCED CHANGES IN VAGINAL CYTOLOGY: MATERIALS ADMINISTERED IN 2 INJECTIONS SEPARATED BY 24 HOURS

Dose (μ g.)		N	+ Response	Percentages of + response
Estrone	Progesterone			
1.5	0	119	66	56
1.5	50	26	16	62
1.5	100	26	13	50
1.5	250	26	12	46
1.5	500	26	16	62
1.5	1000	26	13	50

TABLE 8

EFFECT OF PROGESTERONE ON THE VAGINAL RESPONSE TO ESTRONE: MATERIALS ADMINISTERED IN 2 INJECTIONS SEPARATED BY 24 HOURS

Dose (μ g.)		N	+ Response	Percentages of + response	ED ₅₀ (μ g.)
Estrone	Progesterone				
1	0	115	24	21	2.1
2	0	115	53	46	
4	0	115	90	78	
8	0	115	103	90	
1	1000	18	4	22	3.6
2	1000	18	10	56	
4	1000	18	8	44	
8	1000	18	10	56	

TABLE 9

ABSENCE OF A MARKED EFFECT OF PROGESTERONE ON ESTRIOL-INDUCED CHANGES IN VAGINAL CYTOLOGY IN SPAYED, ADULT RATS: MATERIALS ADMINISTERED IN 2 INJECTIONS SEPARATED BY 24 HOURS*

Dose (μ g.)		N	+ Responses	Percentages of + response
Estriol	Progesterone			
100	0	28	25	89
100	30	9	9	100
100	100	30	22	73
100	300	9	7	78
100	1,000	31	23	74
100	3,000	9	6	67
100	10,000	28	21	75

* Data from Edgren and Elton.³³

after treatment and the vaginas were sectioned for histological study. The progesterone produced the expected mucification of the vaginal epithelium (FIGURE 10), but had no effect upon the development of a typical estrogen-stimulated keratinized sheath (FIGURE 11).

Estriol-induced vaginal cornification also appears to be largely unaffected by progesterone given simultaneously (TABLE 9).

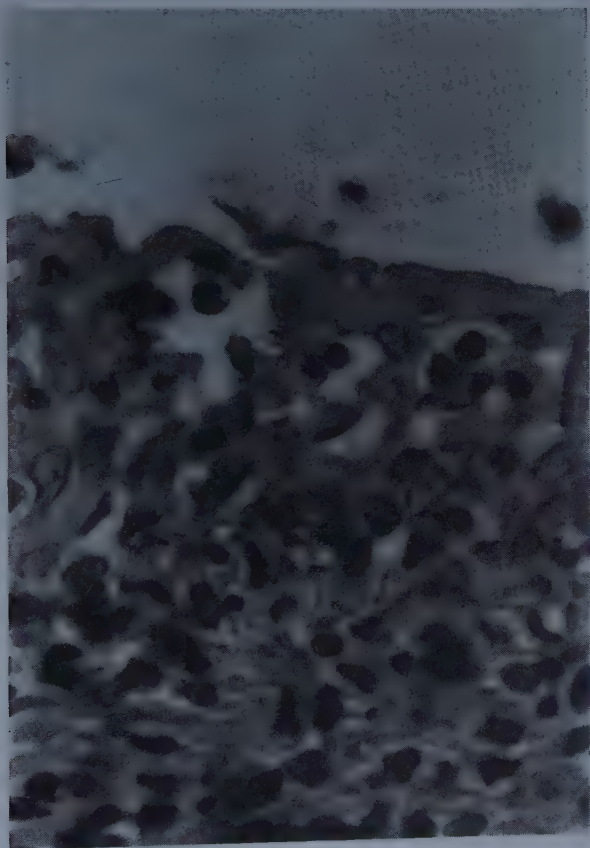


FIGURE 10. Vaginal epithelium of adult spayed rat 48 hours after the administration of 1000 μ g. of progesterone. Note mucification of epithelium.

Effects of Corticoids on the Vaginal Response to Estrogens

For almost 20 years desoxycorticosterone acetate (DCA) has been known to interact with estrogens. Del Castillo and di Paola^{23, 34} demonstrated that this mineralocorticoid decreased vaginal sensitivity to estradiol benzoate in spayed rats, and Robson¹⁴ concluded that its estradiol-inhibiting action in the mouse was not at the vaginal level. In 1948 Szego and Roberts³⁵ showed that stresses, adrenocorticotrophic hormone (ACTH), and DCA inhibited the water-imbibition reaction of rat uteri to administered estradiol; subsequently, Szego ex-

tended these studies to include cortisone and cortisol as estrogen antagonists.³⁶ In rats with hypothalamic lesions the suppression of constant estrus by cortisone was demonstrated by Alloiteau and Courvoisier;³⁷ however, this suppression could have resulted from a pituitary depression rather than from a direct antagonism. Courrier³⁸ showed that estradiol-induced vaginal changes in mon-



FIGURE 11. Vaginal epithelium of adult spayed rat 48 hours after the administration of 10 μ g. of estrone and 1000 μ g. of progesterone. Compare with FIGURE 8.

keys were inhibited by DCA, but not by cortisone, and Takewaki,³⁹ employing spayed rats, showed that both DCA and cortisone inhibited estrone-induced vaginal estrus. Variable responses to mixtures of estrogens and corticoids have been reported.²³

My work with corticoids has been as unimpressive as the studies on progesterone. Cortisone acetate appeared to have no marked effect on a dose of estrone that produced an approximately 70 per cent response (TABLE 10). DCA was ineffective as an antagonist of the vaginal changes produced by 5 μ g. of estrone (TABLE 11).

*Effects of Miscellaneous Nonsteroidal Substances on
the Vaginal Response to Estrogens*

Quite recently there has been considerable interest in the estrogen-antagonistic actions of certain nonsteroidal substances. In 1953 Herschberg and Huidobro⁴⁰ showed that Phenergan and atropine combined, but not individually, would counteract the effects of estradiol on the vagina. Presumably, these substances blocked endogenous histamine and acetylcholine. Bárány and his colleagues⁴¹ have studied the effects of intravaginal instillation of compounds related to the 4,4'-hydroxydiphenylalkanes. These materials were

TABLE 10

EFFECTS OF CORTISONE ACETATE ON ESTRONE-INDUCED CHANGES IN VAGINAL CYTOLOGY:
MATERIALS ADMINISTERED IN 2 INJECTIONS SEPARATED BY 24 HOURS

Dose (μg.)		N	+ Responses	Percentages of + response
Estrone	Cortisone acetate			
1.5	0	15	10	67
1.5	10	8	3	38
1.5	30	15	9	60
1.5	100	15	11	73
1.5	300	15	8	53
1.5	1000	15	7	47

TABLE 11

EFFECTS OF DESOXYCORTICOSTERONE ACETATE ON ESTRONE-INDUCED CHANGES IN VAGINAL
CYTOLOGY: MATERIALS ADMINISTERED IN 2 INJECTIONS SEPARATED BY 24 HOURS

Dose (μg.)		N	+ Responses	Percentages of + response
Estrone	DCA			
5	0	10	10	100
5	100	10	10	100
5	300	10	10	100
5	1000	18	17	94

found to inhibit the vaginal response produced by subcutaneously administered estradiol benzoate. A stilbestrol analogue, 2,3-di-*p*-hydroxyphenylbut-2-ene, referred to as dimethylstilbestrol, was recently shown to antagonize estrogens (estrone, estradiol, or diethylstilbestrol) when both materials were administered intravaginally.⁴² Dimethylstilbestrol was not an estradiol antagonist when either or both compounds were administered subcutaneously; in fact, the data may even have suggested some synergistic action. Since dimethylstilbestrol itself requires a higher intravaginal than subcutaneous dose to produce cornification, it appears to be a proestrogen. Emmens and Cox feel that the active antagonist is the untransformed molecule. In a recent, exhaustive paper Lerner *et al.*⁴³ have explored the biological activities of 1-(*p*-2-diethyl-aminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenyl ethanol (MER-25), which

would appear to be an estrogen antagonist under all circumstances. MER-25 was an antagonist of estradiol-induced vaginal changes in spayed rats whether administered orally or subcutaneously. Similar results also were obtained in monkeys.

Through the kindness of the officials of The Wm. S. Merrell Co., Cincinnati, Ohio, we have had a sample of MER-25 to compare with other antagonists. This material is certainly an antagonist of estrone-induced vaginal changes; however, in comparison with the androgenic steroids, it would not seem to be particularly potent (TABLE 12).

TABLE 12

EFFECTS OF 1-(*p*-2-DIETHYLAMINOETHOXYPHENYL)-1-PHENYL-2-*p*-METHOXYPHENYL ETHANOL (MER-25) ON ESTRONE-INDUCED CHANGES IN VAGINAL CYTOLOGY: MATERIALS ADMINISTERED IN 2 INJECTIONS SEPARATED BY 24 HOURS

Dose (μg.)		N	+ Responses	Percentages of + response
Estrone	MER-25			
5	0	35	26	72
5	500	36	18	50
5	750	16	3	19
5	1000	36	8	22
5	2000	19	1	5

Discussion

The importance of the interactions of steroid hormones remains one of the most enigmatic, but most interesting, problems facing the endocrinologist today. For more than thirty years it has been known that various steroids that occur naturally in the circulation of many species of mammals modify the effects of estrogens. Recent studies on the urinary excretion of steroids demonstrate that urinary titers of the estrogens show remarkable changes throughout the menstrual cycle of humans and during pregnancy.^{44, 45} During the follicular phase of the human cycle estradiol is the dominant estrogen but, as midcycle approaches, estrone becomes more prominent, and the luteal phase of the cycle is largely dominated by estriol; pregnancy, too, is characterized by increasing estriol excretion. Progesterone metabolites show an excretory pattern remarkably similar to that for estriol, and recent work shows that aldosterone increases during pregnancy.⁴⁶ Glucocorticoid production also is known to vary cyclically.⁴⁷ For some time it has been evident from clinical data that reproductive integrity in man is affected greatly by the functional state of the adrenal glands. Thus, we may picture the blood of a normally cycling mammal as a labile, ever-changing pool of steroid hormones.

Meanwhile, experimental studies have shown us that it is difficult to predict how a given steroid will interact with an estrogen in any given experimental approach. With respect to certain pairs of compounds, there are qualitative reversals of effect with changes in dose level in a single assay procedure, that is, estrone-estriol or estrone-16-epiestriol,⁸ estriol-progesterone,^{33, 48} or estrone-testosterone.²² Two other papers in this monograph, those of Rosa and Ve-

lardo and of Wied, give similar data. There are qualitative differences between different assay techniques employing the same target organ, that is, testosterone inhibits estradiol,⁴⁹ testosterone adds to estradiol,⁵⁰ both on rat uterine growth; estriol, estrone, or estradiol are mutually additive,¹ they are mutually inhibitory, vaginal smear studies.⁴² These few examples, which could be multiplied, particularly if tests in which no activity could be found are compared, should suffice to indicate the complexity of the problem (TABLE 13). My associate Calhoun and I have considered this problem for some time and believe that these wide variations in types of reaction may result largely from divergent internal environments with respect to other steroid hormones. The actual reversal of type of activity with differing doses of combinations of two hormones strongly supports this contention.

Thus, it is felt that at any one time any reacting target organ is responding, not to a single substance, but to a shifting pool of steroid hormones that, as

TABLE 13

SUMMARY OF DATA ON THE EFFECTS OF VARIOUS STEROIDS ON THE ACTIONS OF ESTROGENS ON VARIOUS TARGET ORGANS

	Compound					MER-25
	Estrogen	Androgen	Progestin	Corticoids		
				Mineralo-	Gluco-	
Present studies	+	—	0	0	0	—
Other vaginal smear studies	+0—	+0—	+0—	—	—	—
Other target organs	+0—	+0— (44)	+0—	0—	0—	—

Key: —, inhibitory; 0, no effect; +, additive.

proportions change, have both synergistic and antagonistic interactions. My data, much of it unpublished, and the studies of others seem to suggest that the synergisms occur at low dose levels when organs are essentially atrophic, and that antagonisms occur at higher dose levels. These mechanisms would tend to restrict the growth, development, or modification of a target organ within rather narrowly circumscribed limits. If one were considering the vagina and it were under primarily estrogenic control, then if the estrogen fell toward unphysiologically low titers, the vaginal epithelium would be maintained by synergism of the estrogen with other steroids. Unphysiologically high titers of estrogen would be antagonized and the epithelium could not hypertrophy to excessive, perhaps nonfunctional, limits. Studies on the uterus have been particularly convincing in this regard. Calhoun and I both feel that this type of phenomenon is of general applicability, and that it explains the remarkable capacity of female mammals to develop normally and reproduce, despite occasional major insult. We also feel that the explanation for the precise control of female sex cycles lies in some such hypothesis as this. Certainly, the inherent lags and delays in feedback systems make the conventional pituitary-gonad theory of sex-cycle control inadequate without some method of

moderating the effects of these lags. The results of steroid interactions, which on a biological level are a form of "buffering" system, could supply this necessary moderating effect. Thus, we believe that sexual phenomena are controlled by the steroid "buffering" pool, working in conjunction with pituitary-gonad feedback systems and on target organs that are conditioned by other hormonal and nutritional factors.⁵¹

Summary

The effects of combinations of estrogens and of estrogens with various other steroidal agents have been discussed. Interacting materials were separated into five main categories according to their major biological actions. The literature, particularly with respect to the study of combinations of substances on the vagina, has been surveyed for the following categories of substances that interact with the estrogens: other estrogens, androgens, progestins, corticoids, and miscellaneous nonsteroidal agents.

The original work reported was restricted primarily to a single vaginal-smear assay technique, thus allowing reasonably direct comparison among the materials interacting with the estrogens. When estrone was employed in these studies as a standard vaginal stimulator, androgens were the only steroidal antagonists; estrogens augmented the estrone response, whereas progestins and corticoids, both gluco- and mineralo-, had no marked effect. Responses of the estriol-stimulated vagina were similar, although in other tests estrone and estriol were remarkably different.

Histological studies showed that testosterone interfered with the estrogen-stimulated formation of a thick keratinized epithelial sheath lining the lumen of the vagina.

Simultaneous study of vagina and uterus of animals treated with estrone-estriol combinations at dose levels that produced potent inhibition of uterine growth demonstrated that the effects of the combinations on the vagina were not significantly different from the effects of estrone alone. Thus, the site of interaction was localized in the target organ.

The roles of steroid interactions in the endocrine control of sexual development, cycles, and pregnancy are discussed.

Acknowledgments

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Part II. The Human Vagina

THE QUALITATIVE IDENTIFICATION OF FREE AMINO ACIDS IN HUMAN VAGINAL FLUID

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In 1932 Nuernberger⁶ studied the vaginal secretions of 32 unselected females. The protein content varied from 2.0 to 2.5 per cent in normal vaginas, while amounts as great as 6 per cent were found in females with mixed vaginal flora. Glutathione was demonstrated in quantities ranging from 28 to 284 mg. per cent; arginine was the only amino acid identified.

In the past ten years the technique of paper chromatography has been used extensively for the isolation and identification of biochemical components of biological material. The technique is especially suited for study of vaginal amino acids, since chemical compounds present in small quantities may be detected by this procedure.

By paper chromatography a small amount of the material to be studied is placed on a strip of filter paper. The end of the filter paper is then placed in a trough containing an organic solvent that slowly travels along the paper. The components of the mixture move in the same direction as the front of the advancing organic solvent, but less rapidly and each with its own characteristic velocity. A two-dimensional system is possible by turning the filter paper at right angles and utilizing another solvent.

The pattern obtained by one-dimensional chromatography of crude vaginal secretions was studied by Berton² in 1957. In cases of intraepithelial neoplasia of the cervix uteri there appeared to be a decrease or absence of some Ninhydrin-positive compounds. Complicating factors such as menstrual blood, vaginal infection, and the menopause did not seem to alter the basic chromatographic pattern.

By means of two-dimensional chromatography Haour *et al.*⁴ investigated the presence of amino acids in vaginal secretions during pregnancy, the menopause, and various pathological conditions, and in human semen. A total of nineteen amino compounds was found in both genital secretions. The only amino acid identified was glutamic acid. A quantitative difference in amino compounds was demonstrated in different phases of the menstrual cycle, the greatest amount being present in the ovulatory phase. A difference in concentration also was observed before and after estrogen therapy.

None of the above-mentioned workers, however, has attempted to identify and list the various amino acids found.

Materials and Methods

Attempts were made to investigate individual patients for vaginal amino acids. The volume obtained, however, was so small and variable that reproducible chromatograms could not be obtained. Therefore, pooled samples

from five groups of six patients attending the obstetrics and gynecology out-patient clinics of the Jefferson Hospital, Philadelphia, Pa., were obtained with a capillary glass pipette. Small amounts of a thick white fluid ranging in volume from 0.05 to 0.2 ml. were obtained, extracted with 2 ml. of 95 per cent ethyl alcohol, and centrifuged. The alcoholic extract was evaporated to dryness at 50° C. in a water bath under a steady stream of nitrogen; the dried residue was redissolved in an aqueous solution of 10 per cent isopropanol and was chromatographed.

Chromatography was performed with a two-dimensional system of butanol, acetic acid, and water, and of phenol, water, and ammonia. After chromatography the papers were removed, dried in the cold, and sprayed with a 0.1 per cent solution of Ninhydrin. The sprayed chromatogram was heated for 10 min. at 105° C.

One-dimensional chromatograms were utilized to separate amino acids that were then identified by specific chemical reactions. The presence of proline and phenylalanine was tested for by dipping a one-way dimensional chromatogram in isatin. Citrulline and tryptophan were tested for with Ehrlich's reagent,⁷ histidine and tyrosine by the sulfanilic acid reagent.¹ The Sakaguchi reaction was used to identify arginine. Sequential dipping in isatin and Ehrlich's reagent was utilized for the detection of hydroxyproline.⁵ Valine, leucine, and isoleucine were separated and identified in a one-dimensional system of isoamyl alcohol saturated with water.⁸

A map of twenty-two amino compounds that react with Ninhydrin was used as a point of reference in identifying the amino acids.

Results and Discussion

A total of fifteen Ninhydrin-positive compounds was identified in human vaginal fluid. FIGURE 1 demonstrates the pattern obtained in the two-dimensional system. Spot 13 contains leucine and isoleucine, while spot 12 contains valine and tryptophan. Tryptophan was identified by its reaction with Ehrlich's reagent, while leucine, isoleucine, and valine were separated by a one-dimensional system of isoamyl alcohol saturated with water (FIGURE 2). Application of specific tests demonstrated that proline (but not hydroxyproline) was present; histidine (but not tyrosine) gave a positive sulfanilic-acid reaction. No phenylalanine or citrulline was found. The diamino compound glutamine was also present. Although this is not an amino acid per se, it is found distributed in tissues, and is used as a method of storage and removal of amino groups. The amino acids found in vaginal fluid are similar to those obtained in human semen,³ except that tyrosine is not present.

In summary, the following fourteen amino acids were demonstrated to be present in human vaginal secretions: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, proline, serine, taurine, threonine, tryptophan, and valine.

Although the fluid studied was obtained from the vagina, it is not possible to state that it originated solely therein. More controlled investigations are needed to prevent the contamination of vaginal fluids by cervical, uterine, and tubal secretions, as well as by bacterial flora. The role of the amino acids

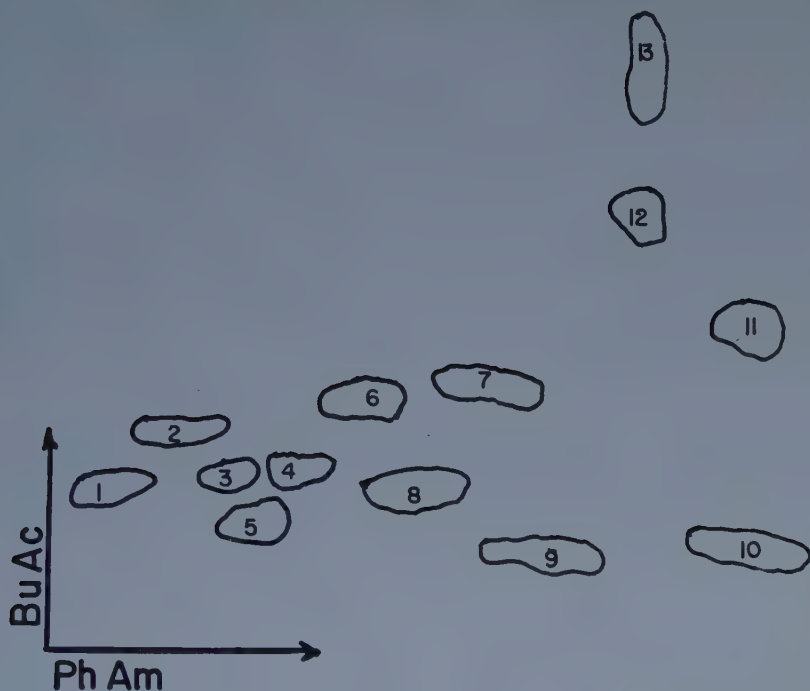


FIGURE 1. Two-dimensional chromatographic pattern of free amino acids found in human vaginal fluid. Solvents used were phenol-ammonia and butanol-acetic acid. Key: 1, aspartic; 2, glutamic; 3, serine; 4, glycine; 5, taurine; 6, threonine; 7, alanine; 8, glutamine; 9, histidine; 10, arginine; 11, proline; 12, valine, tryptophan; and 13, leucine, isoleucine.

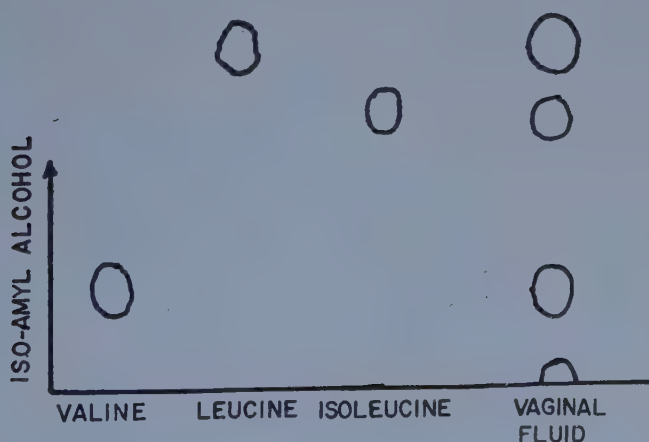


FIGURE 2. One-dimensional chromatographic pattern demonstrating separation of valine, leucine, and isoleucine from human vaginal fluid.

in the process of fertilization and in pathological conditions is unknown and remains to be demonstrated.

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STUDIES ON VAGINAL CONTENTS AND CELLS WITH PROTON MAGNETIC RESONANCE

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There are only a few methods available for examination of the physico-chemical properties of living and relatively intact cells. One such method is nuclear magnetic resonance. This method is a form of spectroscopy using electromagnetic radiation of very long wave lengths (low frequency), which implies that the energy supplied to the biological sample is far beyond any level of damage to the cells subjected to investigation.

The first successful attempts to observe nuclear magnetic resonance in bulk matter were made by Bloch *et al.* (1946) and Purcell *et al.* (1946). Nuclear physics, physical chemistry, organic chemistry, and in the latest years, biochemistry and biology have begun to make extensive use of the nuclear magnetic resonance techniques.

Basic Principles of Nuclear Magnetic Resonance

If a sample containing magnetic atomic nuclei is placed in a strong magnetic field, they will take up certain allowed orientations in the field. This occurs only for nuclei having a spin angular momentum amounting to $\hbar \sqrt{I(I+1)}$, where $\hbar = h/2\pi$ and h is Planck's constant, 6.625×10^{-27} erg/sec. I is the nuclear spin angular momentum quantum number or nuclear "spin." If $I = 0$, the nucleus in question has no associated magnetic moment and will not orient itself in the field. If I is a number such as $\frac{1}{2}$, 1, or $\frac{3}{2}$, the nucleus also possesses a magnetic moment, and will orient itself with respect to the magnetic field.

Quantum theory now predicts that a nucleus having the spin of I can take up $(2I+1)$ permitted orientations in a magnetic field. For example, consider the common and important hydrogen nucleus or proton which will be the subject of nuclear resonance in the present work. It has a spin of $\frac{1}{2}$, and therefore each hydrogen nucleus will take up one of two possible orientations if a sample containing protons (for example, water) is placed between the two poles of a magnet. This situation is illustrated in FIGURE 1.

The magnetic moment is expressed in units of nuclear magneton (μ_0) 5.05×10^{-24} erg/gauss. If a nucleus having the magnetic moment μ (erg/gauss) is placed in a magnetic field of strength H gauss, then the magnetic energy of interaction between the nucleus and the magnetic field is given by the following equation:

$$E = \vec{\mu} \times \vec{H} = -\vec{\mu} \times \vec{H} \cos \theta = -\mu H m/I \text{ (erg)} \quad (1)$$

Consider two neighboring, allowed nuclear orientations. The energy difference, ΔE , between these states is easily obtained from EQUATION 1. Thus

$$\Delta E = \mu \cdot H/I \ (\Delta m = 1) \text{ (erg)} \quad (2)$$

as is illustrated in FIGURE 1. This equation is based upon the assumption that the magnetic quantum number changes with one unit, and it is therefore experimentally possible to induce transitions between these two energy states if the sample is irradiated with electromagnetic radiation of the energy

$$h\nu = \Delta E \text{ (erg)} \quad (3)$$

or frequency

$$\nu = \mu H / I h \text{ (sec.}^{-1}\text{)} \quad (4)$$

EQUATION 4 is the fundamental equation underlying any experiment of nuclear magnetic resonance.

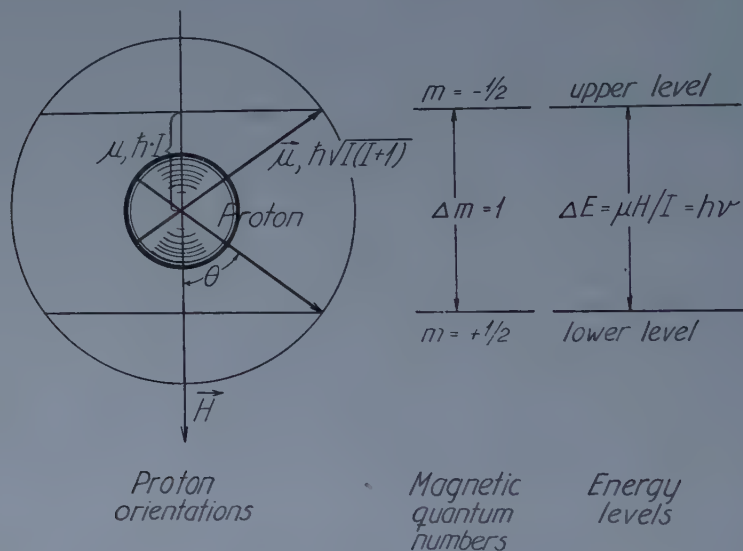


FIGURE 1. Diagrammatic representation of the orientations of nuclear spin and magnetic moment, the magnetic quantum numbers, and energy levels of a proton in a magnetic field. The correct angles of the spin vector orientations with respect to the field H are given. The figure illustrates the meaning of $\vec{\mu}$ and μ . (The value of magnetic moment usually given in tables is μ/μ_0 , μ_0 being the nuclear magneton.) The figure shows that the difference Δm between the two proton orientations is unity. The figure also shows the energy difference ΔE between the two proton orientations.

Several books are referred to for more detailed information on nuclear magnetic resonance (Ramsey, 1953; Grivet, 1955; Andrew, 1955; and Roberts, 1959).

Observation of Proton Magnetic Resonance

Consider a sample of water in a magnetic field of 4930 gauss (the field used in the present study). The magnetic moment of the proton is known to be 2.793 nuclear magnetons. EQUATION 4 predicts that the transitions between the two allowed states of proton orientations will take place if the samples are irradiated with a radiation having the frequency of 21.0 Mc./sec. This radiation is supplied by a coil in a tuned circuit. Suppose that this frequency is kept

fixed and the resonance condition is swept through slowly by changing the magnetic field gradually; then a resonance will be noted just as the field at the hydrogen nucleus passes exactly at 4930 gauss.

The way to observe when the resonance condition occurs can vary in different experimental arrangements. The two inventors of this field mentioned above, Bloch and Purcell, worked out two different techniques that have been denoted nuclear induction and nuclear magnetic resonance absorption, respectively. Which technique is used is a matter of personal preference.

In order to observe nuclear magnetic resonance using Purcell's technique, it is customary to use a radio-frequency bridge. At resonance, imbalance occurs in the bridge, and a signal can be observed after amplification and rectification.

The primary nuclear resonance signal usually is extremely faint, and the electronic equipment therefore should be of very good quality. It is, in addition, necessary to use a very homogeneous magnetic field so that the resonance signal is narrow enough and clearly resolved above electronic noise. The magnetic field preferably should be homogeneous at the order of 1 part in 10 million. In our case, this corresponds to resonance lines having a width of $0.1 \times 10^{-6} \times 21 \text{ Mc./sec.} = 2 \text{ cps.}$ Alternatively, the line width can be expressed as $0.1 \times 10^{-6} \times 4930 \text{ gauss} = 0.5 \text{ milligauss.}$ Finally, the line width can be given as 0.1 ppm. As proposed by Bloch (1954), rotation of a sample will average out many types of field inhomogeneities and make it possible to reach this high degree or a still higher degree of resolution.

Relaxation Times

Some of the protons, the total number of which in the sample is N , occupy the lower one of the allowed orientations and the remainder occupy the higher level (FIGURE 1). The lower energy level is somewhat more populated by protons than the higher energy level. The populations are given by the Boltzmann's distribution, so that the difference between the populations, ΔN , out of the total number of protons, N , is given by the approximate equation

$$\Delta N/N = 2 \mu H/kT \quad (5)$$

where k is the Boltzmann's constant, $1.38 \times 10^{-6} \text{ erg/degree}$, and T is temperature in degrees Kelvin. The introduction of numerical values in this equation gives (for 4930 gauss) approximately $\Delta N/N = 3 \times 10^{-6}$. This is the excess population in the lower energy state, and it is this slight excess that really makes net absorption of radiation possible.

If this small excess is completely removed by intense irradiation from the coil, so that both energy levels become equally populated, no more net absorption can occur and no signal will be observed. Experiments show that such a "saturated" condition does not last very long. Some of the nuclei in the higher energy state lose their energy to the surroundings and return to the lower energy state until Boltzmann's equilibrium is reached. This is the process of thermal relaxation, which is characterized by a time constant expressing the inverse probability of that energy loss. This time constant is called the spin-lattice relaxation time or longitudinal relaxation time, or T_1 . In liquids, the most important mechanisms of spin-lattice relaxation are provided by the

rotational or translational oscillations of neighboring molecules, arising from the thermal agitation.

Exchange of magnetic energy between two neighboring nuclei also occurs, so that one spin flips down at the same moment as the other flips up. Furthermore, this process is characterized by a time constant, the spin-spin interaction time, or transverse relaxation time, or T_2 .

T_1 and T_2 depend strongly on the viscosity of the sample examined, as shown by Bloembergen *et al.* (1948). For common temperatures (about 300° K.) the

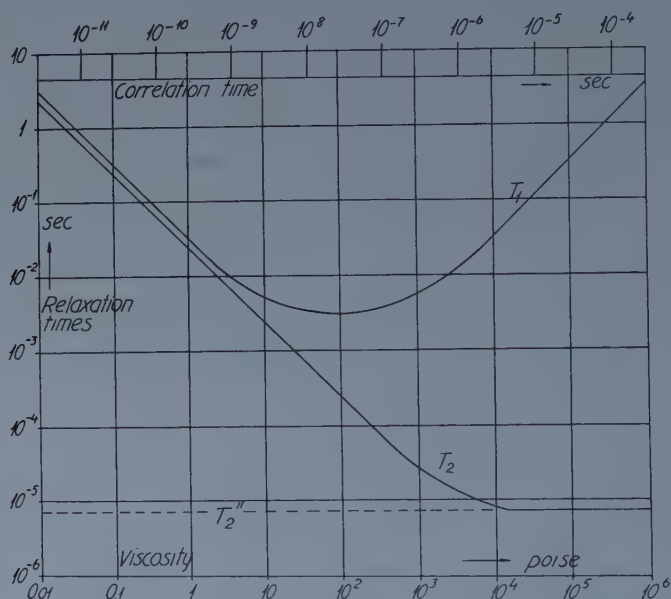


FIGURE 2. Dependency of T_1 and T_2 for protons in water on the viscosity β , and on correlation time, t_c . The normal viscosity of water is 0.01 poise, and T_1 and T_2 are a few seconds. If the viscosity and correlation time are increased, both T_1 and T_2 decrease up to a viscosity of about 10^2 poise (t_c being about the angular frequency of the applied radiofrequency 10^{-8} sec. $^{-1}$). At still higher viscosity, T_1 will rise again. When the viscosity is so high (10^5 to 10^6 poise) that a solid state is reached, having very long correlation times, T_1 will again be on the order of seconds and T_2 will be extremely short.

dependence of T_1 and T_2 on viscosity is indicated in FIGURE 2. It can be seen that, up to viscosities in the range of 100 poise, T_1 and T_2 decrease in a similar manner. At higher viscosities T_1 will rise again, but T_2 will decrease to a minimum value.

A more basic interpretation of the relaxation times is to put them in relation to the correlation times of the sample. The correlation time, t_c , is the average time during which a given molecular arrangement persists. For low viscosities T_1 is inversely proportional to t_c , while for high viscosities T_1 will rise proportionally with t_c . At low and medium viscosity T_2 varies inversely with t_c while, for high viscosities, T_2 is independent of t_c . These relations are shown graphically in FIGURE 2. The correlation time of a molecule in aqueous solution

is given approximately by the equation

$$t_c = V \times \beta / kT \quad (6)$$

where V is molecular volume (in cc.) and β is viscosity (in poise). Several correlation times may exist in a solution, for example, in the interior of a cell.

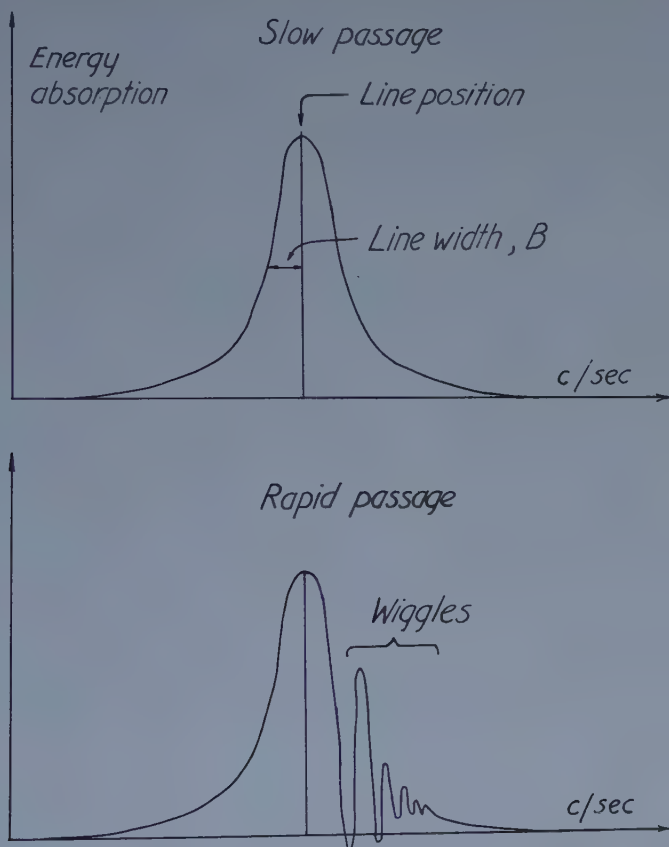


FIGURE 3. Definition of line width B (upper curve). The lower curve illustrates the shape of the nuclear magnetic resonance signal when the signal is recorded very rapidly. The interference effects are called wiggles, and occur if T_2 is relatively long.

T_2 (almost identical with the average lifetime of spin orientations) influences also the width of the resonance signal. The line width B expressed in cps (FIGURE 3) is given by the equation

$$B = \frac{1}{2\pi T_2} \quad (7)$$

which implies that broad resonance lines should exist in highly viscous samples. Factors other than T_2 also may broaden the resonance lines. Confluent unresolved closely lying resonance lines may appear as a single broad line.

Position of the Resonance Signal

The exact position of the resonance line of a sample depends on the chemical form of the protons contained in the sample. The magnetic field H in EQUATION 4 is equal to

$$H = H_{appl} (1 + S \times \chi + \sigma)$$

where H_{appl} is the applied bulk magnetic field, χ is the bulk diamagnetic susceptibility of the sample (of the order of -10^{-6} c.g.s. units) and S a shape factor depending on the shape of the sample, being $0 - 4\pi$. The constant σ is the internal diamagnetic shielding that is exerted by the electrons surrounding the protons and is of the order of 20×10^{-6} . Both χ and σ depend on the chemical structure of the sample. For a sample of pure acetic acid two reso-

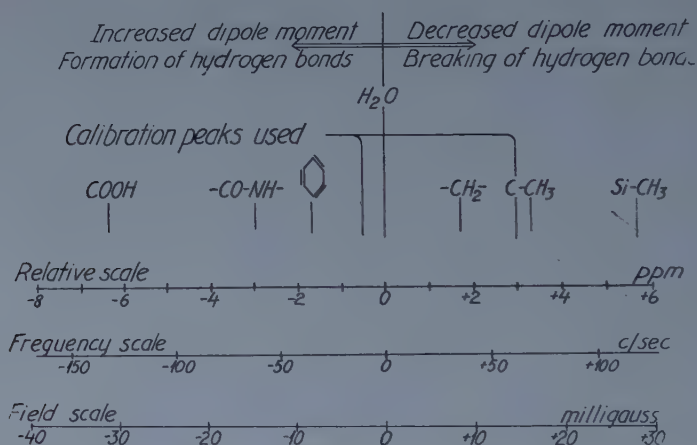


FIGURE 4. The chemical shifts for protons in some different chemical positions, pure water being put as zero reference. The scales are given as ppm, cps, and milligauss (the scales are valid for 21 Mc./sec. only).

nance peaks are noted, one at -6 ppm and one at $+3.5$ ppm (from water reference) due to different internal diamagnetic shielding of carboxyl and methyl protons. Some information on such chemical shifts is collected in FIGURE 4. Detailed studies have been made, for example, by Meyer *et al.* (1953). Hydrogen bonding is known to affect the position. Increased formation of hydrogen bonds shifts the resonance to the negative sign and vice versa. In water, increase of the induced part of the electric dipole moment of the water molecule also will shift the resonance toward lower fields and vice versa (Shoolery and Alder, 1955).

Proton Magnetic Resonance in Biological Material

In complex biological material, as in cell suspensions, several other important factors influence the proton magnetic resonance signal. The occurrence of irregular cell and nuclear membranes introduces complicated shape factors resulting in line broadening. There are present paramagnetic compounds

such as oxygen and various enzymes that have magnetic moments of the order of size of several thousand nuclear magnetons due to the presence of unpaired electrons. These magnetic compounds may strongly influence the spin-lattice and spin-spin relaxation times (Chiarotti *et al.*, 1955, and others).

Paramagnetic compounds also may cause resonance shifts (Dickinson, 1951). The viscosity of the cytoplasm and extracellular fluid also influences the relaxation times and line widths. The water in a biological sample is expected to give its own resonance line, which should dominate the spectrum. Several organic compounds dissolved in the intra- or extracellular water are expected to give separate small resonance peaks. Solid organic material may give rise to one or several broad lines which should be observable after exchange of H_2O with D_2O . (Deuterium has the spin $I = 1$ and a magnetic moment of 0.857 nuclear magnetons, so that it gives resonance lines very far away from the proton resonances.) As mentioned above, changes in the position of the proton resonance line could occur due to breakdown or increase of hydrogen bondings or electric dipole interactions. The presence of water bound to the proteins is expected to increase the correlation time and consequently influence the relaxation times and increase the line width. This is shown in the large series of investigations conducted by Shaw *et al.* (1951 to 1955). The effect by nucleic acids on proton resonance of water has been studied by Jacobson *et al.* (1954). Odeblad and Lindström (1955) studied the proton resonances at low resolution in some tissues. Odeblad *et al.* (1956) made some observations on the proton resonance in red blood cells and Odeblad and Bryhn (1957) preliminarily observed the proton resonance in the human cervical mucus. Huggert and Odeblad (1959) have studied the proton resonance lines of different tissues of the eye, and also especially studied the proton resonance of the crystalline lens. Of large importance is the work by Jardetsky and Jardetsky (1958) on the resonance spectra of amino acids. Their attempt to interpret the ribonuclease spectrum (1957) is also very useful.

Experimental Methods Used

Clinical material. Healthy young women were selected for this study (TABLE 1) at routine health gynecologic examinations. In order to exclude admixture of contraceptive agents and sperm in the samples, only virgins were accepted for sampling of material. The phase of the ovarian cycle was determined in several ways. The women recorded their basal body temperatures during the menstrual cycles during which the investigations were undertaken. Several clinical findings and several properties of the cervical mucus also served to establish the phase. Vaginal smears were taken from the right fornix, and were fixed in ethanol-ether and stained according to Papanicolaou.

Sampling. About 0.5 ml. of endovaginal contents from the upper two thirds of the vagina was removed in a capillary tube by suction. The material was immediately suspended in physiological saline solution and was subjected to gentle stirring for short periods (2 min.) 10 times during 2 hours. The cell suspension was then centrifuged at 230 g for 10 min. The sediment of vaginal cells was transferred to a nuclear magnetic resonance tube having an inner diameter of about 0.1 cm. and centrifuged at 230 g to constant volume of the

cell sediment (time varying from 15 to 120 min.). The tube was sealed by melting, and was then introduced in the magnetic resonance spectrometer.

In some cases part of the vaginal content was transferred into physiological saline solution composed of 97 or 99.3 per cent heavy water (D_2O instead of H_2O), the procedure otherwise being the same.

Sometimes samples of native vaginal contents were introduced in sample tubes and examined in the spectrometer (TABLE 1). Specimens of cervical mucus were also recorded in the spectrometer.

TABLE 1
CASES AND SAMPLES

Case	Age	Number of cycles studied	Number of samples studied			
			Vaginal cell sediments	Native vaginal content	Cervical samples*	Total
A.L.	19	1	6	1	6	13
B.Se.	23	2	4	2	12	18
B.Si.	18	1	2	—	7	9
E.K.	14	3	—	6	21	27
G.E.	17	1	6	1	15	22
I.T.	17	2	—	4	15	19
K.B.	19	1	6	—	7	13
K.W.	20	1	2	—	8	10
M.B.	17	2	4	—	16	20
M.H.	25	1	2	—	5	7
M.L.	20	2	11	—	14	25
M.K.	16	3	—	7	19	26
Total	—	20	43	21	145	207

* This material will form the basis of another study.

Nuclear Magnetic Resonance Technique

The spectrometer described by Lindström and Bhar (1956) and somewhat modified was used. The magnetic field was 4930 gauss and the frequency 21.0 Mc./sec. The double T-bridge described in that paper was employed (FIGURE 5). In the double resonance experiments mentioned briefly in the *Discussion* in this paper, a second coil was applied outside the bridge coil and was supplied with the appropriate frequency.

Determination of position and signal. The tube containing the sample was introduced centrally in an external tube of about 0.18 cm. ID. The external tube contained a mixture of 20 ml. H_2O and 30 ml. CH_3COOH (mole fractions 0.853 and 0.147, respectively). This calibration enabled the determination of the position with a standard deviation of about ± 0.03 ppm. Six to 12 records were made, so that a standard error of ± 0.01 or ± 0.02 ppm was assigned to each recorded experimental point. The records of the resonance signals also contained side bands due to interference effects with sample rotation (FIGURE 6). This technique was used for vaginal cell sediments, native vaginal contents, and cervical mucus.

When observing the very broad signals in D_2O exchanged samples, the sam-

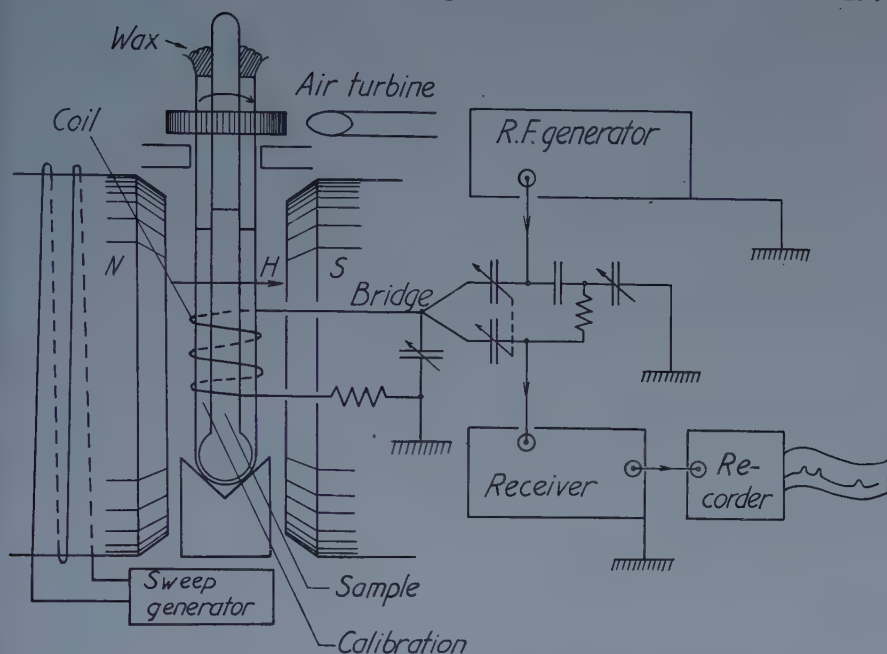


FIGURE 5. Block diagram of the arrangement used for proton magnetic resonance studies. The magnetic poles are *N* and *S*, and the field of the magnet gap is *H*. The magnetic field can be changed with the aid of the sweep generator. The radiofrequency (R.F.) generator supplies energy to the bridge in which the sample is introduced inside a glass test tube containing calibration substance. The sample is rotated with the air turbine. From the bridge the R.F. energy passes to the radio receiver and then to the recorder for graphic registration. The relative sizes of the various components shown may be ignored.

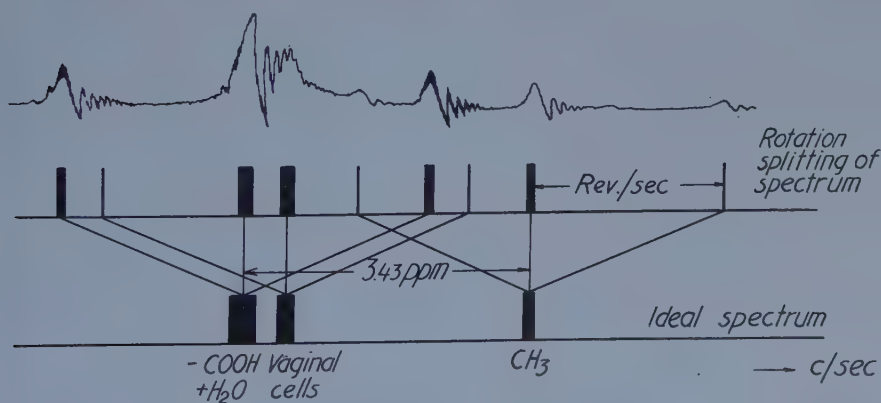


FIGURE 6. An example of a nuclear magnetic resonance signal of a vaginal cell sample, together with a schematic interpretation showing the resonance lines and the rotation splitting. The side bands due to rotation splittings are greater for the calibration substance than for the cell sample because the tube with the calibration substance is broader. On a cps (frequency) scale, the splitting is the number of revolutions/sec.

Case K.B.

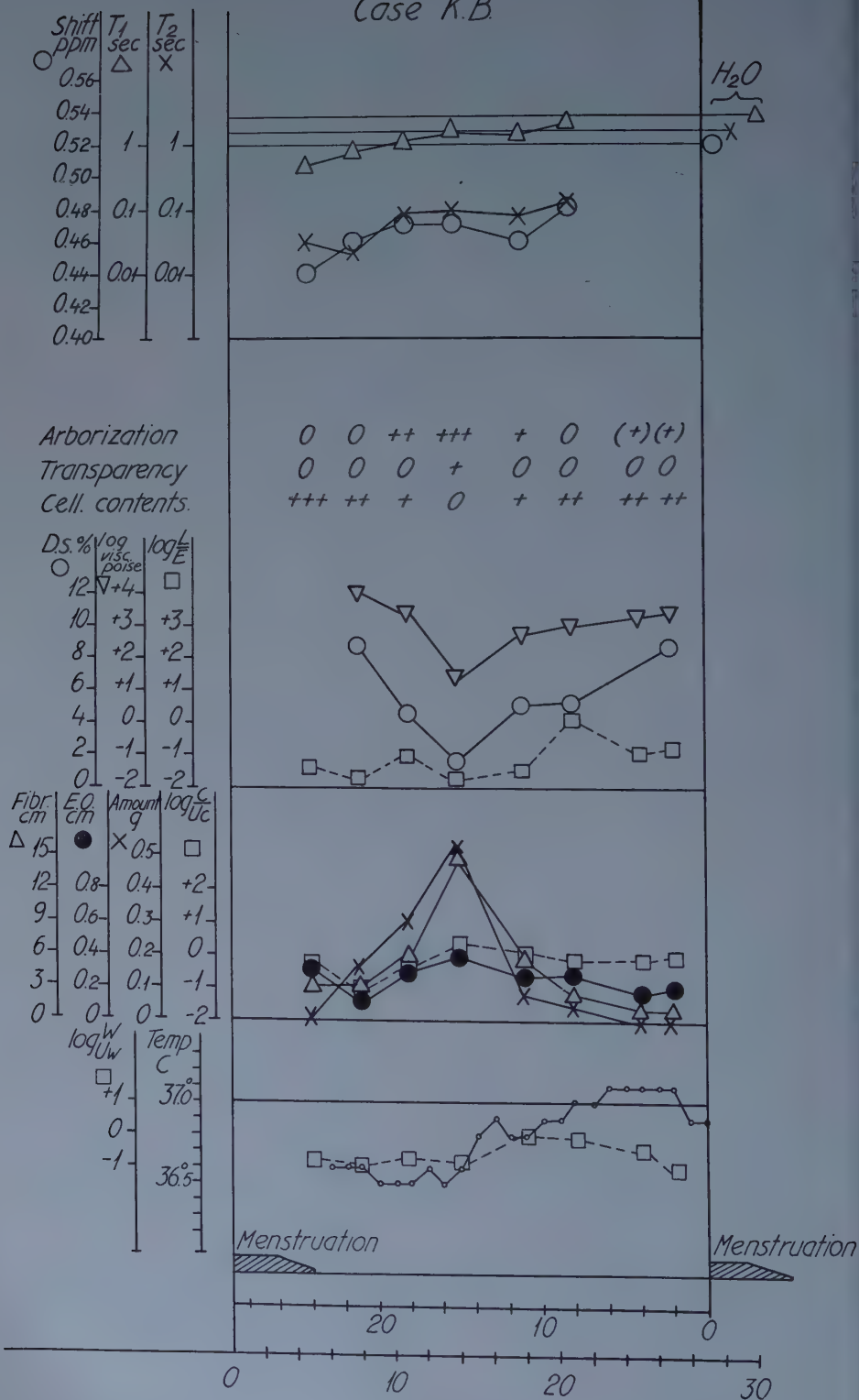


FIGURE 7.

ples were contained in a 0.18 cm. ID tube, and a narrow calibration was sometimes inserted centrally after recordings were made without a calibration tube. Some signals were recorded under saturation of R.F. energy.

Measurement of signal size. The signal size was measured relative to the calibration signals of acetic acid-water. All the corresponding side bands (Williams and Gutowsky, 1956) must be induced in the measurements. The size was expressed as percentage of the size of a water signal. The accuracy was low, about ± 7 per cent.

Measurement of signal width. The signal width (defined in FIGURE 3) was easily measured either on the records containing the calibration signals or in separate recordings without calibration.

Measurement of T_1 . T_1 was measured by observing signal growth after a saturation signal had been applied.

Measurement of T_2 . T_2 was measured with the technique of Gabillard (1951), using repetition of signals and measurement of decay and growth of wiggles.

Results

The proton resonance spectra and relaxation times of vaginal cell sediments could be followed more or less completely during 20 menstrual cycles, 2 of which are shown in FIGURES 7 and 8. Numerous data on the cervical mucus, vaginal smears, and other clinical findings are also presented, offering the possibility of an accurate determination of the phase of the menstrual cycle. The position of the proton resonance signal was localized on the negative side of that for water, and showed a tendency to increase from about -0.10 ppm postmenstrually to about 0.02 ppm (water reference) at the height of the luteal phase. The T_1 values were around 0.6 to 1 sec. during the entire menstrual cycle, while the T_2 values were about 0.03 sec. at the beginning of the menstrual

FIGURE 7. Some proton magnetic resonance data on vaginal cell sample of case K.B. during a menstrual cycle, together with a number of other measurements for determination of phases of the menstrual cycle. The following explanations apply to both FIGURES 7 and 8. At the bottom are given the time scales and the menstrual data (and the occurrence of intermenstrual pain, if any). Higher in the diagram is shown the basal body temperature, together with the occurrence of wrinkled epithelial cells in the vaginal smear. Both curves are expected to be low except during the luteal phase. The occurrence of wrinkled cells is given as the logarithm of the quotient (number of wrinkled cells/number of unwrinkled cells), in the figure denoted $\log W/Uw$. On the next level of the diagram there are collected four properties having the common ability to reach maximum at the peak of midcycle estrogen secretion from the ovary. They are the diameter of external os (E.o.), the amount of cervical mucus (Amount), the fibrosity of cervical mucus (Fibr.), and the occurrence of cornified epithelial cells of the vaginal smear, also expressed as a logarithm $\log C/Uc$ as explained above. At a still higher level of the diagram three properties, all having the common behavior of showing a minimum at the time of maximum midcycle excretion of estrogen from the ovary, are given. The properties are the following: the viscosity of the cervical mucus given logarithmically ($\log \text{visc.}$), the percentage of dry substance of the cervical mucus (D.s.) and the occurrence of leukocytes in the vaginal smear [also expressed as a logarithm, $\log L/E$ of the quotient (leukocytes/epithelial cells)]. Higher up are collected three qualitative properties of the cervical mucus, namely the arborization, the transparency, and the cell contents. All these data taken together will give good information on the phase of the menstrual cycle. At the top of the diagram are given the position of the proton resonance line, the spin lattice relaxation time, T_1 , and the spin-spin interaction time, T_2 , of the cell sediments. The position is given in ppm from the carboxyl peak in a mixture of acetic acid and water, as is described in text. With this calibration the position of pure water is at $+0.52 \pm 0.004$ ppm. The normal values of T_1 and T_2 for water are also given.

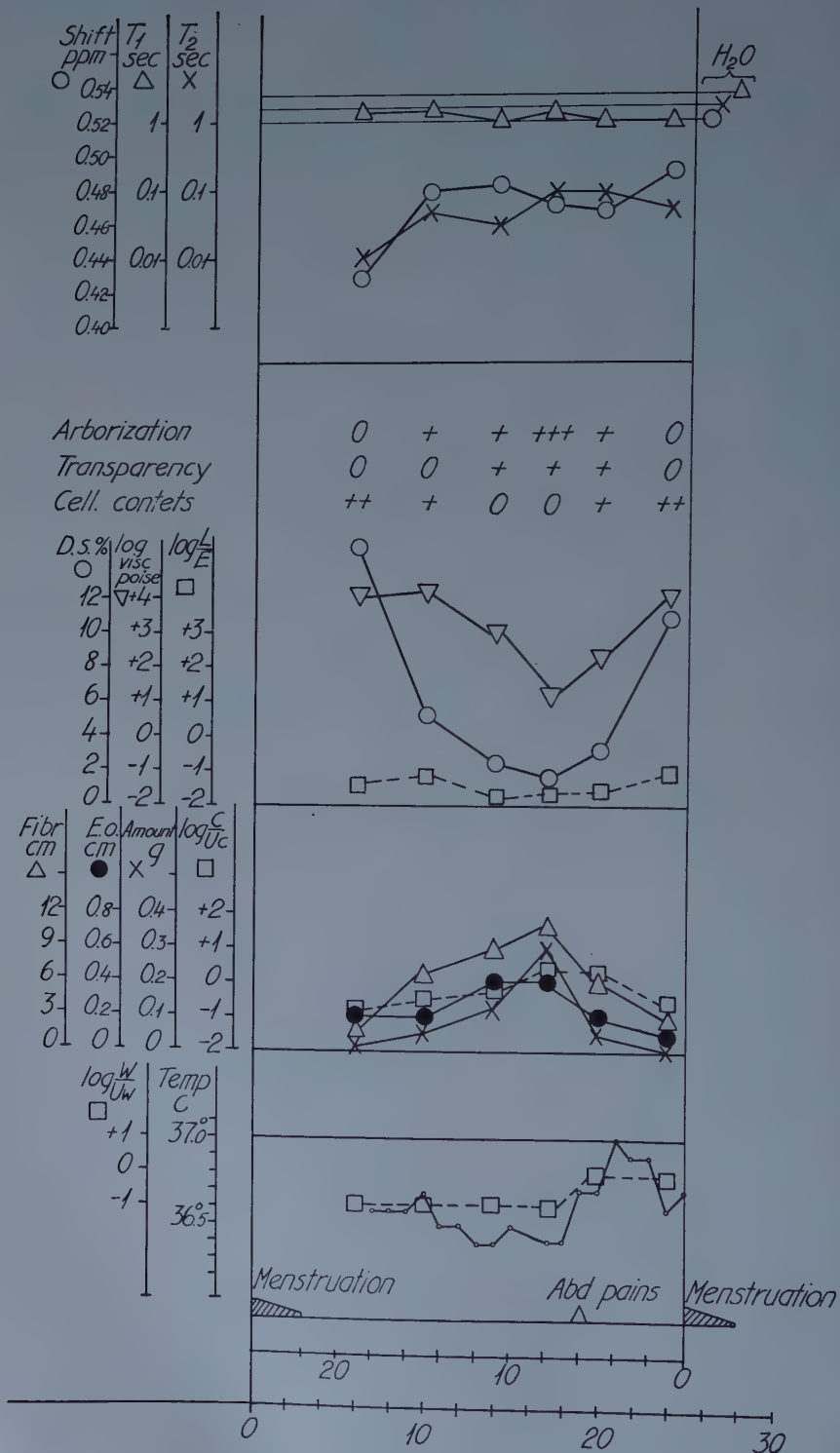


FIGURE 8. A diagram similar to that in FIGURE 7, for case M.L., Cycle I; see FIGURE 7 for explanation.

cycle, with a tendency to increase to about 0.10 sec. as the menstrual cycle reached the height of the luteal phase. There was a significant difference between T_1 and T_2 .

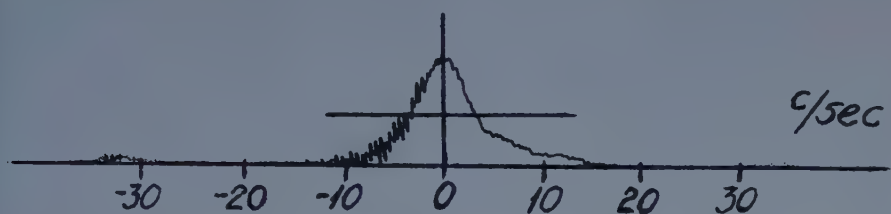


FIGURE 9. Experimental resonance line from case M.L., showing the line width to be 3.1 cps or $T_2 = 0.05$ sec. T_2 , measured by wiggle decay and growth, was 0.06 sec.

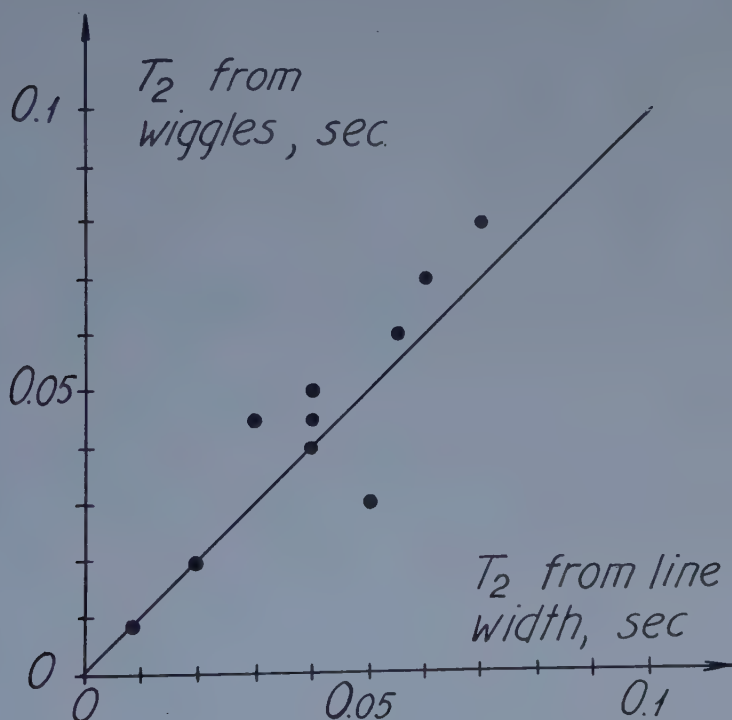


FIGURE 10. Relation between T_2 calculated from line width on wiggles in 10 cases.

The areas of the resonance signals could be determined with an accuracy of about ± 7 per cent, and were nearly constant during the cycle, being about 80 per cent of the expected area for a water signal. The line widths are exemplified in FIGURE 9, and showed a tendency to decrease as the cycle proceeded. There was a definite relation between T_2 calculated from the width and T_2 obtained from the wiggle regrowth method (FIGURE 10).

If the results are compiled according to the probable endocrine stimulus action on the vaginal mucosa, the results shown in TABLE 2 are obtained.

TABLE 2
NUCLEAR RESONANCE DATA AT VARIOUS PROBABLE ENDOCRINE STIMULATIONS

	Position (ppm from H ₂ O)	Signal size (percentage of water line area)	Signal width (cps)	T ₂ (sec.)	T ₁ (sec.)
Postmenstrual phase (low estrogenic stimulation)	-0.10	70	8	0.02	0.5
Midcycle (high estrogenic stimulation)	-0.04	60	7	0.02	0.7
Premenstrually (moderate estrogenic + high progestational stimulation)	-0.02	80	3	0.06	1.0

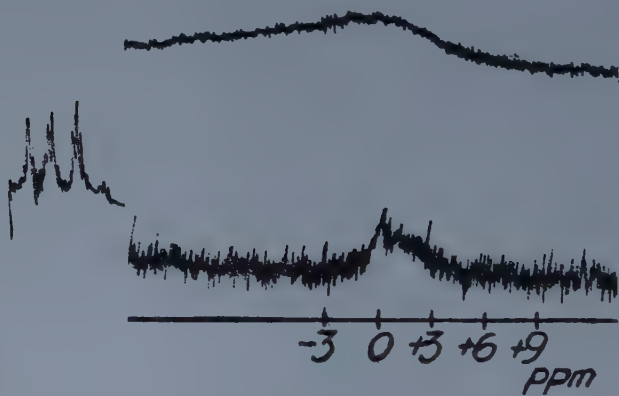


FIGURE 11. Proton resonance of D₂O- (98 per cent) exchanged cells of case K.B. The upper trace is the signal at ordinary conditions, and the lower trace is under saturation of R.F. energy. The narrow peak has then disappeared (*T*₁ being long), while the broad peak remains (*T*₁ being short). To the left there is a signal of ethyl alcohol for calibration of scale

TABLE 3
PROTON RESONANCE DATA ON VAGINAL CELL SAMPLES EXCHANGED WITH D₂O

Case	Phase	Narrow component (of position 0)			Broad component				
		T ₁ (sec.)	Width (cps)	T ₂ (sec.)	Position	T ₁ (sec.)	Width (cps)	T ₂ (sec.)	Signal area
K.B.	Postmenstrual	—	—	—	+2	0.05	60	0.003	—
M.H.	Postmenstrual	—	5	0.03	+1.5	—	33	0.005	0.05
G.E.	Postmenstrual	3	3	0.05	+2	0.1	40	0.004	0.02
G.E.	Midcycle	1	4.5	0.04	+3	—	32	0.005	0.04
K.W.	Midcycle	1	8	0.02	+2.5	—	15	0.01	0.03
A.L.	Early luteal	—	—	—	+3	0.05	80	0.002	—
M.B.	Luteal	1	5	—	+3	—	20	0.008	0.02

In vaginal cell samples subjected to exchange with D_2O , the exact position was very difficult to determine because of the large line width (FIGURE 11). The position appeared, however, to be about +2 ppm from water reference. Some information on T_1 was obtained from the progressive saturation method and by measurements as described above and on T_2 from the line width. The relaxation times appear to be short, or of the order of milliseconds (TABLE 3).

Sometimes there was strong evidence of the presence of two components in T_1 in samples which were exchanged with D_2O . It seems possible to explain these findings with the assumption that the short T_1 component comes from nonexchangeable protons and the long component from the protons of the easily exchangeable cell water. The bulk magnetic susceptibility of some cell sediment was measured by a resonance method and found to be -0.72 ± 0.01 c.g.s. units. The results of the preliminary double-irradiation experiments will be mentioned below.

Discussion

The results indicate that the proton resonances of the vaginal cell sediments deviate significantly from the magnetic resonance properties of water. The negative shift would indicate the presence of increased hydrogen bonding of the water of the cells or increased electric dipole interaction of the water molecules. The short values of T_1 and especially T_2 indicate a high degree of hydration, especially during the first phase and midphase of the menstrual cycle. The adsorption of water on the surfaces of the protein complexes may be responsible for increased hydrogen bonding. Increased dipole interaction could take place in the neighborhood of charged points of the organic molecular chains. It is also possible that carboxyl groups in accumulated organic acids in the vaginal cells (such as lactic acid, β -oxybutyric acid, and others) could be responsible for at least part of the negative shift observed. About 0.15 millimoles per ml. of organic acids may be required to account for 0.01 ppm of negative shift.

As regards the observed difference between T_1 and T_2 , this could be explained by assuming an appropriate degree of admixture of a solid state to the liquid state of the water. This admixture of solid state should represent the water which is strongly absorbed on the solid constituents of the cells. It is, however, interesting to note that other mechanisms may be responsible for the observed phenomenon. Zimmerman (1954) and Laukien and Schlüter (1956) have observed that T_1/T_2 is significantly greater than unity for watery solutions of paramagnetic ions, a phenomenon also reported by Hausser and Laukien (1957). Bloembergen (1957) has offered an explanation for this phenomenon. Meiboom *et al.* (1957) have observed and interpreted the fact that $T_2 < T_1$ also in pure water on the basis of exchange between free and hydrogen-bonded states of the proton. It is not possible to decide at present which of these or other possible mechanisms is responsible for the observed fact that $T_2 < T_1$ in the vaginal cell samples.

The organic unexchangeable ("solid") matter which is visualized after D_2O exchange is characterized by very short values of T_1 and T_2 . The values are, however, not typical for a "solid body" in the physical sense, because the values

of T_1 are too low and the T_2 values are too high to be representative for a pure solid state. The best explanation is that the "solid" constituents execute considerable molecular movements (rotations, oscillations, or translations) giving rise to rather short correlation times capable of explaining the observed values of T_1 and T_2 .

The data of uncentrifugated vaginal contents are usually very close to those of the cervical mucus, and it seems to be necessary that the vaginal cells be separated by centrifugation to be freed from effects from the cervical secretion.

One important question is to decide how much of the content in the samples is composed of vaginal cells. Attempts were made to perform cell countings and measurement of cell volumes. The cell volume of the cornified cells was estimated at 10^{-9} cc., a value based on dimension studies on suspended cells. The cell countings on the samples subjected to magnetic resonance usually showed about 3×10^8 cells per cc. Cell countings of acceptable accuracy were possible only at midcycle, when the clumping of cells was least pronounced. The figures imply that only about 30 per cent of the sample volume was occupied by cells.

Another and more accurate way to study this question was to label the suspension medium with Na^{22} , which is believed to penetrate into the cells only to a small degree. This method gives values of about 40 per cent total cell volume for the cell suspensions.

A third method was to measure the percentage of dry substance in the cell sediments. This percentage was found to be between 15 and 20, while the expected figure for a pure cell sample may be about 25 per cent, indicating approximately 60 per cent total cell volume.

It is evident that part of the cell suspensions was occupied by extracellular fluid. In spite of this finding only one aqueous component of the resonance signals usually could be identified; also, the width agreed with the T_2 value determined by the wiggle method. This might indicate rapid exchange of water across the cell membrane. In a few instances two overlapping components could be resolved (FIGURE 12). They were separated by about 0.6 ppm, and their relative size may indicate that the component at lower field is extracellular fluid. Investigations are now under way on this problem.

If the reasonable value of 5×10^{-5} sq. cm. as the value of the cell surface is assumed, it is found that one hydration layer of water molecules should occupy only about 0.1 per cent of the total sample volume. Thus there may be a mechanism that makes possible the arrangement of the order of, for example, 10^2 layers of water molecules on the cell surfaces. One possible explanation for that type of water binding may be the presence of mucinous substances on the cell surfaces, deriving either from the cervical mucus or from the cells themselves (Botella-Llusia and Nogales, 1955).

One of the most interesting developments of nuclear magnetic resonance is the double-irradiation or double-resonance method. Without going into details, it may be said that this method can give valuable information on the bond mechanisms. I have observed the proton resonances under simultaneous intense irradiation on D_2O -exchanged cell suspensions at N^{14} resonance frequency (1.51 Mc./sec.) or deuterium resonance frequency (3.19 Mc./sec.). So far, no effects were observed on the proton spectra or relaxation times, indicating

very weak preliminary coupling between the irradiated and observed nuclei. These types of experiments are now in progress, using irradiation of D^2 , N^{14} , and P^{31} , as well as other developments of the nuclear resonance studies on vaginal cell sediments.

The proton magnetic resonance studies performed have in no way solved the problems of the physicochemical conditions prevailing in the vaginal epithelial cells. It may be said, however, that information has been obtained which is very difficult to obtain in other ways. The most important experience is, perhaps, that this method has been shown to work on the cell material and has indicated a way for future approach to certain problems related to the physiology of cells and tissues.

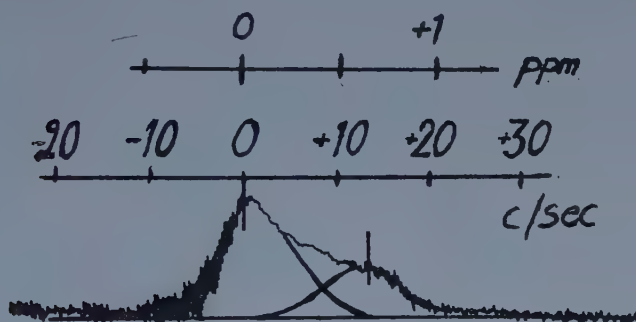


FIGURE 12. Proton resonance of a vaginal cell sediment of case Y.A., in which the signal is very suggestive of the presence of two components. After exchange with D_2O , none of the components could be found, so that it is certain that both are due to water. The fractional areas of the two components are, respectively, 0.72 and 0.28. The fractional extra- and intracellular spaces for Na^{22} were 0.74 and 0.26. The peaks were separated by 13 cps, 0.6 ppm, or 3 milligauss.

Summary

The essentials of the proton magnetic resonance technique are described. This nondestructive method has been applied to the study of the properties of vaginal contents and cell sediments. The positions of the resonances, their size, and their width were measured, and the proton relaxation times T_1 and T_2 were determined.

The position was about -0.10 ppm from water reference at the beginning of the cycle, and increased to about -0.0 premenstrually. The size agreed with the water content, and the width decreased. T_1 was about 0.8 sec.; T_2 was about 0.06 sec., with a tendency to increase as the cycle proceeded.

Exchange with D_2O revealed a weak and broad component located at about $+2.5$ ppm, T_1 being less than 0.05 sec. and T_2 about 0.004 sec.

The results are discussed in relation to the hydration of water associated with the vaginal epithelial cells and the structure of the solid constituents of the cells.

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SYNERGISM AND ANTAGONISM OF SEX STEROIDS AS DETERMINED ON THE VAGINAL EPITHELIAL CELLS

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Estrogens, androgens, or progestogens induce either proliferation of the vaginal epithelium or regressive changes, or they have no effect on the stage of proliferation.¹ The type of epithelial reaction to a given hormonal stimulus is greatly dependent upon the stage of proliferation at the time of administration, the endocrinological condition of the patient, and individual variations in epithelial response.² Cytological determinations of the specific epithelial response to administered steroids may be used in determining the proper mixture of estrogens and androgens, or estrogens and progestogens. The cytological response, however, is not always useful in determining the actual clinical effect of the substance,³ especially in dealing with androgens or progestogens.²

Material and Method

For the present study, seven surgical castrates were used. These showed a constant atrophic epithelial pattern in their vaginal smears prior to the administration of the steroids.

There are physiological variations in the height of proliferation even in surgical castrates. Under "constant" atrophic vaginal epithelia, only those cases were included that showed only an atrophic smear pattern (FIGURE 1) during an observation period of six weeks prior to the first test administration of steroids. Surgical castrates exhibiting even slight variability in their height of epithelial proliferation (FIGURE 2) during the pretest period were not included. The castrates then received, prior to the actual tests, an adequate proliferation dosage of estrogens to induce growth of the epithelium. A period of waiting ensued until epithelial atrophy returned. Castrates in whom atrophy did not return after a reasonable period of time were excluded from the tests. The castrates received estrogens, androgens, and progestogens in separate administrations. These test cycles always were separated by reasonable time intervals in order to permit the epithelium to regress. Later, estrogens plus androgens, estrogens plus progestogens, and finally estrogens plus androgens plus progestogens were administered. These administrations also were separated by reasonable time intervals in order to permit the epithelium to regress. The individual dosages and types of steroids are shown in the respective tables, or in the section entitled *Results*.

The cytological response was determined from 300 squamous cells in each smear by means of cytological indices: (1) the karyopyknotic index, which expresses the ratio of superficial cells to intermediate cells, (2) the eosinophilic index, which expresses the ratio of eosinophilic superficial or intermediate cells to cyanophilic superficial or intermediate cells, (3) the crowded cell index, which expresses the ratio of cells lying in clusters of less than four cells, and (4) the folded cell index, which expresses the ratio of folded superficial or intermediate cells to flat superficial or intermediate cells. It was felt that the

evaluation of only one index, such as the karyopyknotic index, would not be significant enough,⁴ since slight increases of the karyopyknotic index alone may sometimes be observed after administration of some types of androgens.

The cytological terminology used is the one derived from the opinion poll of the International Academy of Gynecological Cytology, Buffalo, N. Y.,⁵ that is, all large polygonal squamous epithelial cells containing a pyknotic nucleus are called superficial cells, regardless of their staining reaction, and all polygonal squamous epithelial cells containing a nonpyknotic (vesicular) nucleus (other

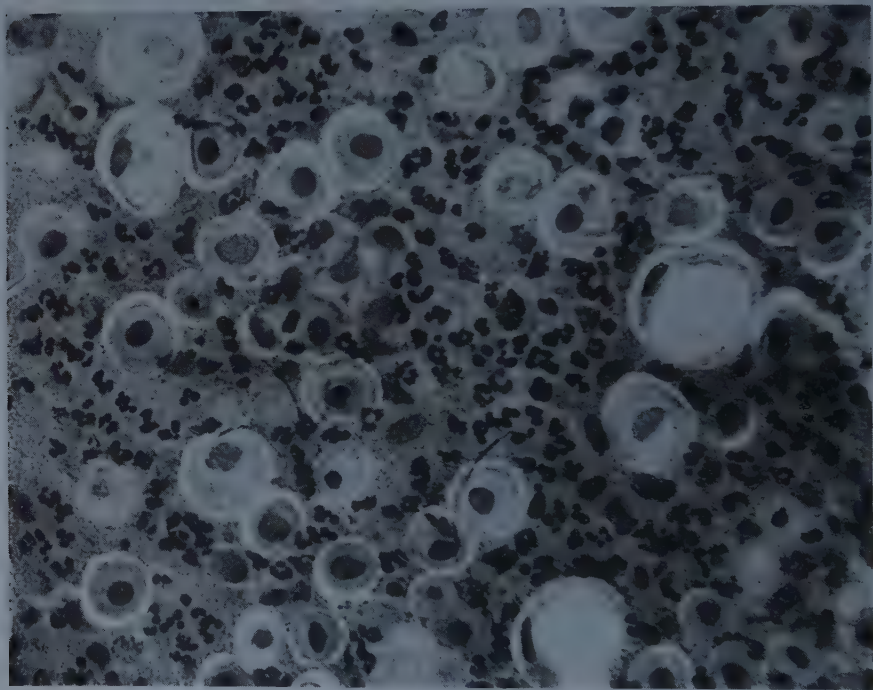


FIGURE 1.

than the so-called parabasal-basal cells) are called intermediate cells, regardless of their staining reaction. The terms acidophilic and basophilic are replaced by eosinophilic and cyanophilic, respectively.

A stained nucleus was determined arbitrarily as pyknotic if it was sufficiently dense to deviate the light of the phasemicroscope (Zeiss Winkel phasemicroscope, 10 $\times$ obj.), giving the impression of a bright red nucleus.^{6,7} Nuclei that appeared dark under the phasemicroscope were not classified as pyknotic, regardless of whether or not they appeared rather dense under the normal bright field microscope. This standard was introduced to permit an objective criterion for the so-called pyknotic nucleus. It was felt that it was more important to use a method permitting high reliability than to argue over the exact meaning of the term pyknotic.

Results

Androgens alone. Androgens administered parenterally to seven surgical castrates with an initial atrophic epithelial pattern of the vaginal smear (FIGURE 1), induced slight or moderate epithelial growth in all seven cases.

The androgens used were: (1) testosterone propionate (6 daily I.M. injections, 50 mg. each); (2) testosterone enanthate (1 I.M. injection, 200 mg.); and (3) testosterone cyclopentylpropionate (1 I.M. injection, 200 mg.).

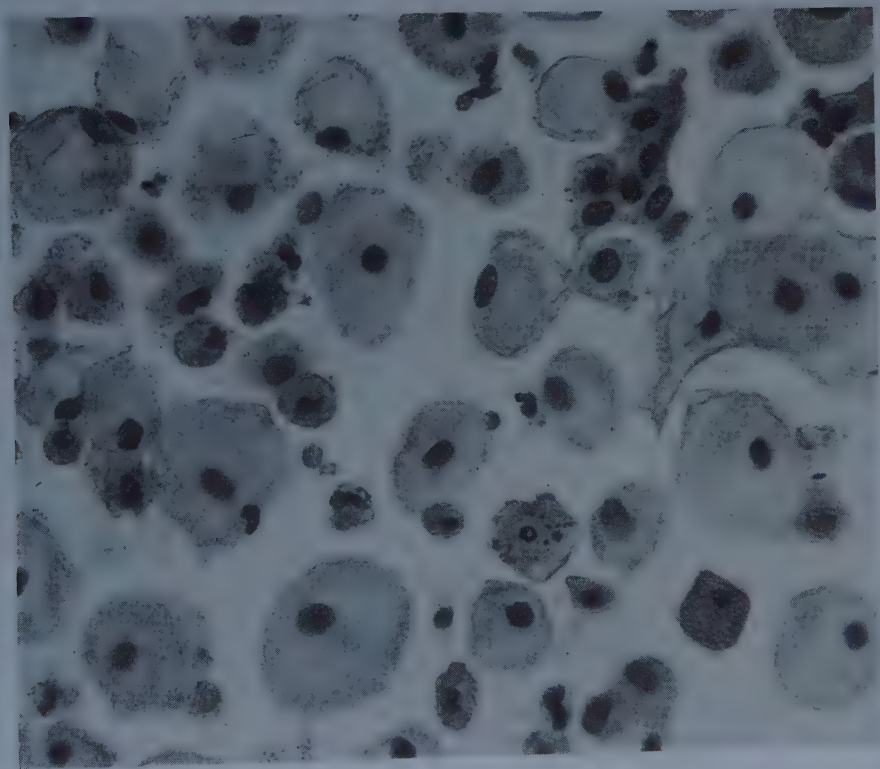


FIGURE 2.

The minimal response to any of the above administrations was:

Parabasal-basal cells	82 per cent	} (prior to administration 100 per cent)	
Intermediate cells	18 per cent		
Superficial cells	0 per cent		
			folded cell index 100 per cent
			crowded cell index 33 per cent
			eosinophilic index 0 per cent

The maximal response (FIGURE 3) to any of the above administrations was:

Parabasal-basal cells	1 per cent	} (prior to administration 100 per cent)	
Intermediate cells	94 per cent		
Superficial cells	5 per cent		
			folded cell index 87 per cent
			crowded cell index 48 per cent
			eosinophilic index 3 per cent

The average response to the above administrations was:

Parabasal-basal cells	32 per cent	(prior to administration 100 per cent)	
Intermediate cells	64 per cent		
			folded cell index 90 per cent
			crowded cell index 66 per cent
			eosinophilic index 0.5 per cent
Superficial cells	2 per cent		

Progestogens alone. Progestogens administered parenterally to 7 surgical castrates with an initial atrophic epithelial pattern of the vaginal smear (FIGURE 1) induced less uniform cellular growth than previously observed in the andro-

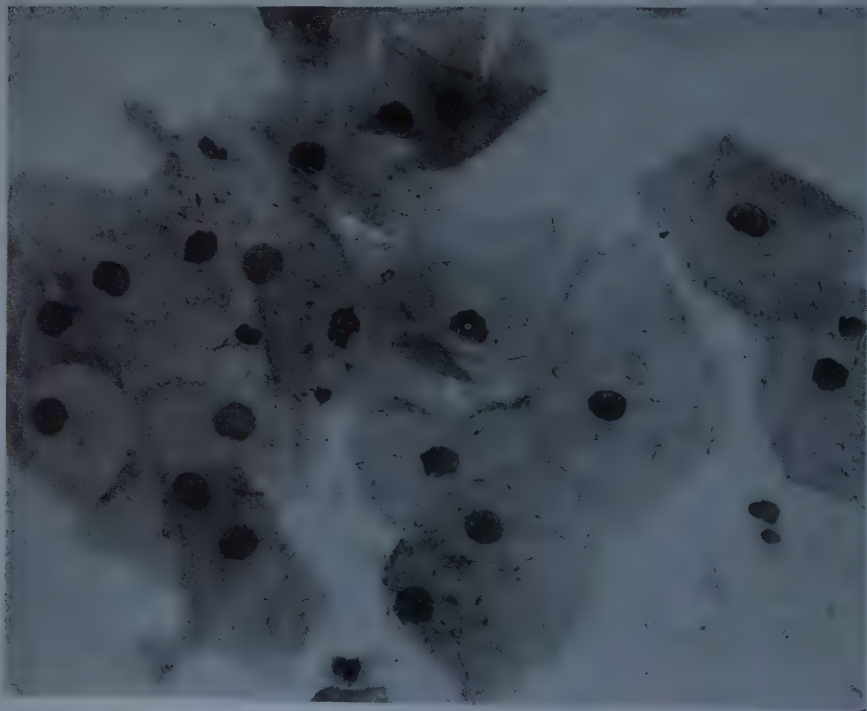


FIGURE 3.

gen tests. In 2 of the 7 surgical castrates no epithelial response was found. In the remaining 5 cases, the growth was rather heterogeneous: a few superficial cells, together with some intermediate cells and many parabasal-basal cells.

The progestogens used were: (1) progesterone (6 daily I.M. injections, 50 mg. each); (2) 17 α -OH-progesterone caproate (1 I.M. injection, 375 mg.); and (3) 6-methyl-17 α -OH-progesterone acetate (1 I.M. injection, 200 mg.).

The minimal response in the five cases which did show some response was:

Parabasal-basal cells	86 per cent	(prior to administration 100 per cent)	
Intermediate cells	13 per cent		
			folded cell index 98 per cent
			crowded cell index 10 per cent
			eosinophilic index 0 per cent
Superficial cells	1 per cent		

The maximal response (FIGURE 4) to any of the above administrations was:

Parabasal-basal cells	14 per cent	(prior to administration 100 per cent)	
Intermediate cells	80 per cent		
			folded cell index 75 per cent
			crowded cell index 31 per cent
			eosinophilic index 1 per cent
Superficial cells	6 per cent		

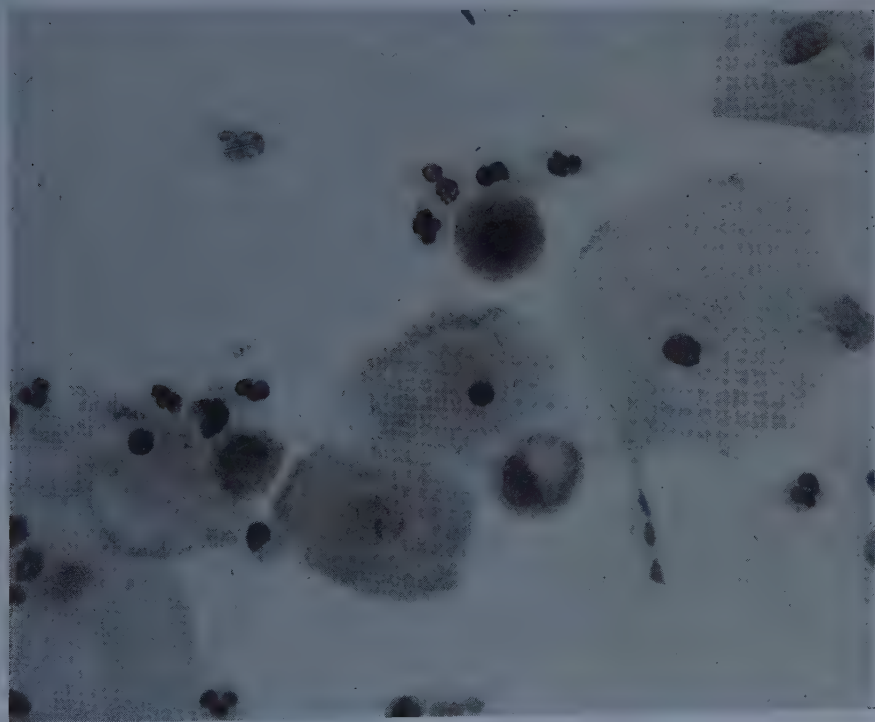


FIGURE 4.

The average response to the above administrations (on all seven castrates) was:

Parabasal-basal cells	58 per cent	(prior to administration 100 per cent)	
Intermediate cells	40 per cent		
			folded cell index 82 per cent
			crowded cell index 28 per cent
			eosinophilic index 0.2 per cent
Superficial cells	2 per cent		

Estrogens alone. Estrogens administered parenterally to seven surgical castrates with an initial atrophic epithelial pattern (FIGURE 1), induced marked proliferation of a homogeneous cell pattern in all cases.

The estrogens used were: (1) estradiol benzoate (6 daily I.M. injections, 2.5 mg. each); (2) estradiol valerate (1 I.M. injection, 10 mg.); and (3) estradiol cyclopentylpropionate (1 I.M. injection, 10 mg.).

The minimal response to any of the above administrations was:

Parabasal-basal cells	4 per cent	(prior to administration 100 per cent)
Intermediate cells	84 per cent	
		}
Superficial cells	12 per cent	}
		folded cell index 72 per cent
		crowded cell index 28 per cent
		eosinophilic index 2 per cent

The maximal response (FIGURE 5) to any of the above administrations was:

Parabasal-basal cells	0 per cent	(prior to administration 100 per cent)
Intermediate cells	28 per cent	
		}
Superficial cells	72 per cent	}
		folded cell index 17 per cent
		crowded cell index 0 per cent
		eosinophilic index 42 per cent

The average response to the above administrations was:

Parabasal-basal cells	0.5 per cent	(prior to administration 100 per cent)	
Intermediate cells	58.5 per cent		
		} folded cell index 38 per cent	
			crowded cell index 6 per cent
Superficial cells	41.0 per cent		

Estrogen-androgen mixtures. Estrogens and androgens together were administered intramuscularly to the seven surgical castrates. The substances

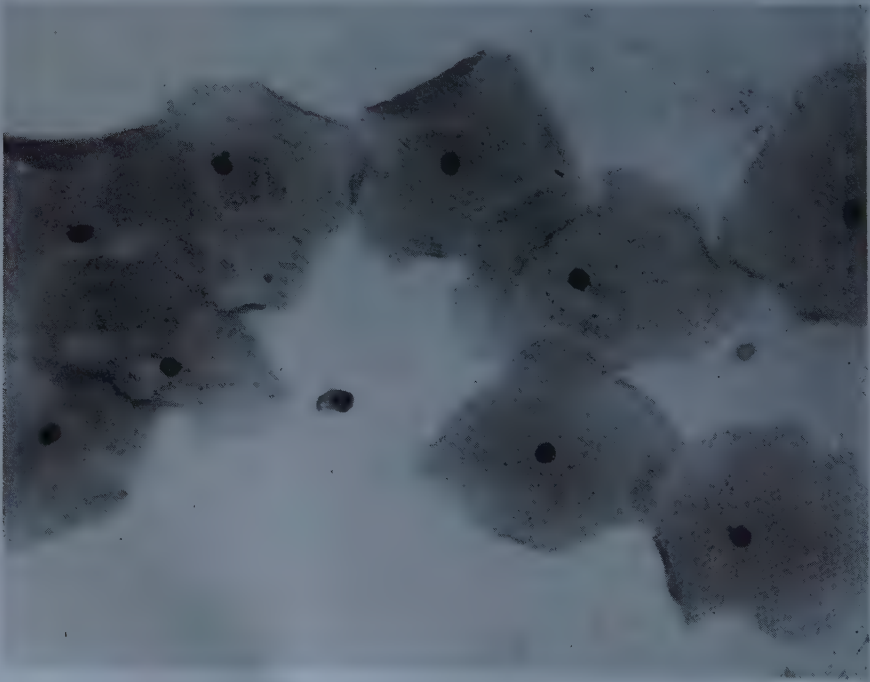


FIGURE 5.

used were estradiol cyclopentylpropionate and testosterone cyclopentylpropionate in various mixtures (TABLE 1).

The mixture of 1 to 20 is apparently sufficient to suppress the occurrence of the characteristic estrogenic pattern, as far as parenterally administered estradiol cyclopentylpropionate and testosterone cyclopentylpropionate are concerned.

TABLE 1

Estradiol cyclopentyl- propionate	Testosterone cyclopentylpropionate			
	0	50 mg.	100 mg.	200 mg.
	Percentages			
0		Androgenic prol. Parab.C. 38 K.I. 2 E.I. 0 F.C.I. 82 C.C.I. 38	Androgenic prol. Parab.C. 37 K.I. 3 E.I. 1 F.C.I. 87 C.C.I. 42	Androgenic prol. Parab.C. 33 K.I. 2 E.I. 0 F.C.I. 90 C.C.I. 55
2.5 mg.	Estrogenic Parab.C. 9 K.I. 10 E.I. 2 F.C.I. 42 C.C.I. 19	Suppression Parab.C. 0 K.I. 3 E.I. 1 F.C.I. 69 C.C.I. 27	Suppression Parab.C. 2 K.I. 2 E.I. 0 F.C.I. 77 C.C.I. 39	not performed
5 mg.	Estrogenic Parab.C. 2 K.I. 18 E.I. 11 F.C.I. 32 C.C.I. 10	Estrogenic Parab.C. 0 K.I. 17 E.I. 10 F.C.I. 30 C.C.I. 12	Suppression Parab.C. 1 K.I. 2 E.I. 1 F.C.I. 63 C.C.I. 32	Suppression Parab.C. 0 K.I. 1 E.I. 0 F.C.I. 72 C.C.I. 39
10 mg.	Estrogenic Parab.C. 0 K.I. 33 E.I. 12 F.C.I. 35 C.C.I. 6	Estrogenic Parab.C. 0 K.I. 35 E.I. 13 F.C.I. 33 C.C.I. 8	Estrogenic Parab.C. 0 K.I. 14 E.I. 9 F.C.I. 29 C.C.I. 5	Suppression Parab.C. 0 K.I. 3 E.I. 1 F.C.I. 67 C.C.I. 19
20 mg.	Estrogenic Parab.C. 0 K.I. 38 E.I. 20 F.C.I. 20 C.C.I. 8	not performed	not performed	Estrogenic Parab.C. 0 K.I. 18 E.I. 12 F.C.I. 28 C.C.I. 6

As an interesting observation it was noticed that there were still some parabasal-basal cells present after administration of 2.5 mg. estradiol cyclopentylpropionate alone, but there were no parabasal-basal cells present after administration of the combination of 2.5 mg. estradiol cyclopentylpropionate with 50 mg. testosterone cyclopentylpropionate. In this particular case estrogens and androgens apparently both acted synergetically (inducing disappearance of the parabasal cells) and antagonistically (inducing regression of the height of proliferation).

The combination of 1 to 10 clearly resulted in estrogenic effects, acting as though no androgens had been added to the estrogens.

Estrogen-progesterone mixtures. Estrogens and progestogens were administered together in intramuscular injections to the seven surgical castrates. The substances used were estradiol valerate and 17α -OH-progesterone caproate in various mixtures (TABLE 2).

TABLE 2

Estradiol valerate	17α-hydroxyprogesterone caproate							
	0		250 mg.		375 mg.		500 mg.	
	Percentages							
0			Parab.C.	38	Parab.C.	41	Parab.C.	35
			K.I.	3	K.I.	2	K.I.	4
			E.I.	0	E.I.	0	E.I.	1
			F.C.I.	82	F.C.I.	73	F.C.I.	89
			C.C.I.	12	C.C.I.	22	C.C.I.	19
2.5 mg.	Estrogenic				not performed			not performed
	Parab.C.	8	Parab.C.	3				
	K.I.	9	K.I.	4				
	E.I.	3	E.I.	1				
	F.C.I.	32	F.C.I.	58				
	C.C.I.	12	C.C.I.	27				
5 mg.	Estrogenic							not performed
	Parab.C.	2	Parab.C.	1	Parab.C.	2		
	K.I.	14	K.I.	2	K.I.	1		
	E.I.	10	E.I.	0	E.I.	0		
	F.C.I.	29	F.C.I.	69	F.C.I.	70		
	C.C.I.	9	C.C.I.	39	C.C.I.	43		
10 mg.	Estrogenic		Estrogenic					
	Parab.C.	0	Parab.C.	0	Parab.C.	0	Parab.C.	0
	K.I.	29	K.I.	17	K.I.	9	K.I.	0
	E.I.	14	E.I.	11	E.I.	4	E.I.	0
	F.C.I.	24	F.C.I.	32	F.C.I.	52	F.C.I.	91
	C.C.I.	6	C.C.I.	11	C.C.I.	12	C.C.I.	52
20 mg.	Estrogenic		not performed		Estrogenic			
	Parab.C.	0			Parab.C.	0	Parab.C.	0
	K.I.	35			K.I.	33	K.I.	12
	E.I.	17			E.I.	17	E.I.	8
	F.C.I.	18			F.C.I.	25	F.C.I.	44
	C.C.I.	0			C.C.I.	0	C.C.I.	8

The mixture of 1 to 50 is apparently sufficient to inhibit the occurrence of the typical estrogenic pattern of proliferation. The mixture of 1 to 37 was sufficient to show some suppression, but the mixture of 1 to 25 was either inadequate or resulted only in minimal regressive changes.

Estrogen-androgen-progesterone mixture. Estrogens, androgens, and progestogens were given only in one dosage: 20 mg. estradiol valerate plus 375 mg. 17α -OH-progesterone caproate plus 100 mg. testosterone cyclopentylpropionate.

This dosage was chosen because our objective was to determine whether the

addition of 100 mg. testosterone cyclopentylpropionate would result in suppression of the estrogenic proliferation, as was found previously after the administration of the above estrogen-progestogen mixture.

TABLE 3 shows the previous results and the changes induced after administration of the estrogen-androgen-progestogen mixture.

The previously tested mixture of 200 mg. testosterone cyclopentylpropionate and 20 mg. estradiol cyclopentylpropionate was not sufficient to suppress the occurrence of the estrogenic proliferative cell type. However, individually inadequate dosages of progestogens and androgens administered together were sufficient to suppress the occurrence of the estrogenic cell type. Androgens and progestogens apparently acted synergistically.

TABLE 3

	375 mg. 17 α -OH-progesterone caproate	375 mg. 17 α -OH-progesterone caproate plus 200 mg. testosterone cyclopentylpropionate
	Percentages	
Estradiol valerate, 20 mg.	Estrogenic	Suppression
	Parab.C. 0	Parab.C. 0
	K.I. 33	K.I. 3
	E.I. 17	E.I. 1
	F.C.I. 25	F.C.I. 69
	C.C.I. 0	C.C.I. 32

Conclusions

(1) Androgens alone induce growth of the atrophic epithelium of the surgical castrate and result in exfoliation of a mixture of intermediate and parabasal cells, rarely superficial cyanophilic (practically never eosinophilic) cells.

(2) Progestogens alone sometimes induce growth of the atrophic epithelium of the surgical castrate and result in a more heterogeneous proliferation than observed after the administration of androgens. Many parabasal and intermediate cells, but also some superficial cells, are found.

(3) Estrogens alone induce, in all cases of atrophic epithelium in surgical castrates, marked epithelial proliferation which is clearly distinguished from the one occurring after progestogen administration by its height of proliferation and by its uniform pattern, whereas some low dosages of estrogens may result in proliferative patterns similar to those observed after androgen administration.

(4) Estrogens and androgens administered together act as antagonists, depending upon the dosages of mixture of the steroids. The tested cyclopentylpropionic acid esters of estradiol and testosterone inhibit the occurrence of the estrogenic proliferative cell type if administered in mixtures of approximately 1 to 20.

(5) Estrogens and progestogens administered together act as antagonists, again depending upon the relative dosages of the steroid mixture. The tested mixture of estradiol valerate and 17 α -(OH)-progesterone caproate was found to

result in inhibition of the occurrence of the estrogenic proliferative type if administered in a ratio of approximately 1 to 37 to 1 to 50.

(6) If administered together, androgens and progestogens may act synergistically as antagonists to the estrogens, achieving suppression of the proliferation due to the simultaneously acting estrogens. In a mg./mg. relationship, the combined effective dosage of androgens plus progestogens which was found to suppress the estrogenic proliferation was 1 (estrogen) to 28 (10 androgen + 18 progestogen).

Summary

On cytological vaginal smear studies it was demonstrated to what extent estrogens, androgens, and progestogens act as synergists or antagonists on the vaginal epithelium of the surgical castrate.

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A STUDY OF DÖDERLEIN'S VAGINAL BACILLUS*

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Since the observations and findings of numerous workers tend to indicate that the Döderlein bacillus is a constant and, in some instances, the only inhabitant of the normal vagina, it becomes of interest to gain insight concerning the characterization of this organism. There have been a number of efforts by many to classify this organism, but workers in this field have encountered difficulty in isolating these bacteria from mixed cultures and in maintaining them.

Lash and Kaplan¹ have described the morphology and some cultural reaction of strains of Döderlein's bacillus. These results indicate that this is not one species of bacteria. Thomas² compared several strains of Döderlein bacilli on a morphologic and cultural basis, and showed that they are closely related to *Lactobacillus acidophilus*. Studies by Cruickshank and Sharman³ and by Cruickshank⁴ have emphasized the importance of knowing something concerning the classification of these organisms.

Materials and Methods

In this study 22 cultures tentatively classed as Döderlein bacilli were used. These strains were isolated from patients attending the Gynecology Out-patient Clinic of the Indiana University Medical Center, Indianapolis, Ind. In addition to these strains three pure culture strains of lactobacilli were obtained from the American Type Culture Collection. These strains included *Lactobacillus bulgaricus* strain 9224, *L. acidophilus* (Döderlein's bacillus) strain 9857, and *L. acidophilus* (bifidus) strain 4962.

Several media were used unsuccessfully for the primary isolation of the Döderlein organism. These media included 5 per cent blood agar, Lactalysate agar,‡ and tomato juice agar.§ The media used in this study for the primary isolation of these organisms were phytone‡ dextrose serum broth (PDS) and phytone‡ dextrose serum agar (PDS agar) which were first described by Gilmore.⁵ The components of this medium were as follows: phytone,‡ 2 gm.; dextrose, 1 gm.; distilled water, 90 ml.; and human serum, 10 ml.

The phytone and dextrose were dissolved in the water and the medium tubed in 9-ml. amounts. After sterilization at 121° C. for 15 min., the medium was cooled and 1 ml. of human serum was added to each tube. Agar plates were made by adding 1.5 per cent agar to the above ingredients. The human serum was added just prior to pouring the agar plates. The final pH of the medium varied between 6.0 and 6.5.

During the isolation of the last 9 strains, a medium described by Rogosa *et al.*⁶ was used, lactobacillus selective medium‡ (LBS). It was found that this medium was able to support growth of lactobacilli in comparable numbers

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† Present address: State University of Iowa, Iowa City, Iowa.

‡ Product of Baltimore Biological Laboratories, Baltimore, Md.

§ Product of Difco Laboratories, Detroit, Mich.

and was more efficient in eliminating the growth of extraneous organisms. However, there was some question as to whether all strains of Döderlein bacilli were able to grow on this medium.

Vaginal swabs were inoculated either to PDS broth and PDS agar plates or onto LBS agar plates. The broth tubes were incubated at 37° C. The agar plates were incubated both aerobically and anaerobically at 37° C. All aerobic plates were incubated in an atmosphere of 10 per cent carbon dioxide, 20 per cent oxygen, and 70 per cent nitrogen. Anaerobic conditions were maintained through the use of Brewer jars with an atmosphere of 10 per cent carbon dioxide and 90 per cent nitrogen, or by using oats jars. Pure culture isolates were maintained either on PDS agar slants or in PDS broth tubes, and were transferred at weekly intervals.

Carbohydrate fermentation reactions were studied, using 2 per cent phytone broth and the appropriate carbohydrates added to a 1 per cent final concentration with bromcresol purple as the indicator. Concentrated carbohydrate solutions were sterilized separately and were added aseptically to the tubes of phytone broth immediately prior to inoculation with the organism. All carbohydrate tubes were inoculated with 0.1 ml. of a saline-washed suspension of the organism being tested. The majority of these organisms seemed to do well in 2 per cent phytone plus 1 per cent added carbohydrate, but not as well as with added serum.

As an alternate procedure during the latter part of this study, PDS broth was used for the carbohydrate fermentation reactions substituting the desired carbohydrate for the dextrose called for in the formula. The medium was made up in its entirety and was then sterilized by passage through a Selas filter* prior to inoculation with the organism. It was felt that this procedure would promote maximal growth of the organism.

Catalase determinations were made by adding 3 per cent by volume of 3 per cent hydrogen peroxide to 24-hour broth cultures of the organisms contained in Smith fermentation tubes. An evolution of gas was taken as an indication that catalase was present.

The milk reactions of the various strains of Döderlein bacilli isolated were studied, using skim milk with bromcresol purple as an indicator. The milk tubes were inoculated with 0.1 ml. of a saline-washed suspension of the organism to be tested and then incubated at 37° C. for 10 days. The tubes were examined daily. At the conclusion of the incubation period a pH determination was made, using a Beckman model G pH meter supplied with Beckman No. 1440 electrodes.

The pure culture isolates were examined for Gram-stain reaction, motility, spore formation, cellular morphology, and colonial morphology.

Results

The colonial morphology of the Döderlein bacilli isolated and the morphology of the American Type Culture Collection strains 9857, 9224, and 4962 are given in TABLE 1. As a group, the colonies of the various strains of organisms are practically indistinguishable from one another after 24 hours' incubation on PDS agar. The colonies tend to be small, circular, and convex. The

* Product of Scientific Equipment Division, Selas Corp. of America, Philadelphia, Pa.

diameter of the colonies on PDS agar after 24 hours' incubation is usually not greater than 2 mm. The colonies are white in appearance and are rather soft in consistency. Upon aging, the colonies become dry and mealy and usually assume a dirty white appearance. Upon primary isolation, the majority of these isolates grew better under anaerobic conditions.

The reactions on blood agar for many of these organisms were found to be quite similar. Only strains 9857, 9224, 4962, and 83 produced narrow zones of methemoglobin around the colony. In general, these organisms produce on blood agar tiny punctiform round colonies that seldom exceed 0.5 to 1.0 mm. in diameter. The colonies are difficult to see in transmitted light and are best seen in reflected light.

TABLE 1
MORPHOLOGIC CHARACTERISTICS OF THE DÖDERLEIN BACILLUS

Strain number	Gram stain	Motility	Spores	Size	Cellular morphology
9857*	Positive	negative	none	0.4-0.6 × 3.0-6.0 microns	short chains and filaments
9224*	Positive	negative	none	0.4-0.6 × 2.0-4.0 microns	filaments and beaded forms
4962*	Positive	negative	none	0.8-1.0 × 2.0-6.0 microns	bifid forms and palisade forms
761	Positive	negative	none	0.5-0.7 × 3.0-5.0 microns	chains, filaments, and bifid forms
77	Positive	negative	none	0.5-0.7 × 3.0-4.0 microns	filaments
73	Positive	negative	none	0.8-1.0 × 2.0-4.0 microns	chains
44	Positive	negative	none	0.4-0.6 × 0.8-1.0 microns	filaments
79	Positive	negative	none	0.4-0.6 microns wide	predominantly filaments
83	Positive	negative	none	0.4-0.6 × 2.0-3.0 microns	filaments with roping
483	Positive	negative	none	0.6-0.8 × 1.0-2.0 microns	filaments
78	Positive	negative	none	0.3-0.4 × 0.8-2.0 microns	filaments
60	Positive	negative	none	0.6-0.8 × 1.0-4.0 microns	filaments

* American Type Culture Collection strains of lactobacilli.

The colonial and cellular morphologies of the organisms studied are presented in TABLES 1, 2, 3, and 4. These organisms were found to be extremely pleomorphic in older cultures and had a tendency to lose the Gram reaction after 24 hours' incubation. The cellular arrangement tends to vary from palisade formation to short straight or tightly coiled chains to long straight or slightly wavy chains. An example of this variability in cultural appearances of some of these organisms is shown in FIGURE 1. These photomicrographs were made of Gram stains of 24-hour cultures of the organisms.

Carbohydrate fermentation reactions reveal that these organisms vary from very active fermenters to those having virtually no fermentative ability. This fact is in agreement with the findings of Gilmore,⁵ but contrary to the findings of Thomas.²

The 24- and 48-hour fermentation reactions for these organisms are given in TABLES 5 and 6. Strain 73 presents an interesting fermentation pattern. It will be noted that these organisms do not produce acid from mannitol, sorbitol, dulcitol, salicin, or inositol in 24 hours, but do so in 48 hours.

Glycogen is not fermented by many of these organisms in 48 hours. Other workers have indicated that the Döderlein organisms are slow glycogen fermenters; therefore, it is assumed that incubation periods of longer than 48 hours are necessary in order to attain a more nearly true interpretation of glycogen utilization by these organisms.

In order to study the delayed fermentation reactions of these organisms, the last 12 strains were allowed to incubate for 9 days. The samples were checked

TABLE 2
CHARACTERISTICS OF THE DÖDERLEIN BACILLUS

Strain number	Gram stain	Motility	Spores	Cellular morphology
2-8	Positive	negative	none	irregularly arranged, short, granular, blunt
3-8	Positive	negative	none	wide, blunt rods in curved chains
4-8	Positive	negative	none	short, blunt rods irregularly arranged
5-8	Positive	negative	none	short, blunt rods irregularly arranged
6-8	Positive	negative	none	short, blunt rods in palisades
8-8	Positive	negative	none	blunt rods in short, straight chains
9-8	Positive	negative	none	curved chains
15-8	Positive	negative	none	palisade forms
17-a-a	Positive	negative	none	tightly coiled chains, curved rods
17-b-8	Positive	negative	none	palisades and short filaments
20-8	Positive	negative	none	straight filaments
24-8	Positive	negative	none	palisade forms
30-a-8	Positive	negative	none	short, straight filaments
30-b-8	Positive	negative	none	short, straight and curved chains
31-8	Positive	negative	none	palisade forms
33-a-8	Positive	negative	none	short, straight filaments and palisades
33-b-8	Positive	negative	none	long, curved chains
37-7	Positive	negative	none	palisades and short, straight filaments
39-7	Positive	negative	none	palisades
42-b-7	Positive	negative	none	palisades
43-a-7	Positive	negative	none	palisades
47-7	Positive	negative	none	irregular arrangement

TABLE 3
CULTURAL CHARACTERISTICS OF THE DÖDERLEIN BACILLUS

Strain number	Oxygen relations	Blood agar	Growth on PDS agar slant	Colonial morphology upon PDS agar
9857*	Microaerophilic	greening	filiform	umbonate, dull, gray-white
9224*	Microaerophilic	greening	beaded	umbonate, rugose, dirty-white
4962*	Microaerophilic	greening	beaded	convex, circular, entire, dirty-white
761	Facultatively anaerobic		beaded	convex, circular, entire, dirty-white
77	Anaerobic		filiform	convex, circular, mucoid, white
73	Anaerobic		beaded	convex, entire, dirty-white
44	Microaerophilic		beaded	convex, entire, mucoid, dirty-white
79	Microaerophilic		beaded	punctiform, convex
83	Anaerobic	greening	filiform	umbonate, brittle, dirty-white
483	Microaerophilic		beaded	convex, entire
78	Microaerophilic		filiform	punctiform, white, convex
60	Anaerobic		beaded	convex, entire, punctiform

* American Type Culture Collection strains of lactobacilli.

at intervals of 1, 2, 3, 5, 7, and 9 days. All negative samples were reinoculated with the strain on the fifth day, and allowed to incubate an extra four days. As can be seen by the results in TABLE 6, several strains were slow fermenters, but no characteristic pattern emerged.

The milk reactions for the organisms studied are given in TABLE 7. The final pH in milk after 10 days' incubation varies considerably.

All of the organisms studied were found to be catalase-negative.

TABLE 4
CULTURAL CHARACTERISTICS OF THE DÖDERLEIN BACILLUS

Strain number	Oxygen relations	Colonial morphology upon PDS agar
2-8	Anaerobic	round, convex, shiny, white, opaque
3-8	Anaerobic	round, convex, shiny, white, opaque
4-8	Anaerobic	round, convex, shiny, dirty-white, translucent
5-8	Anaerobic	round, flat, granular, translucent, dirty-white
6-8	Anaerobic	round, flat, granular, translucent, dirty-white
8-8	Anaerobic	round, flat, lumpy, shiny, dirty-white, translucent
9-8	Anaerobic	round, convex, shiny, white, opaque
15-8	Anaerobic	round, convex, shiny, dirty-white, translucent
17-a-a	Anaerobic	round, convex, smooth, white, opaque
17-b-8	Anaerobic	round, convex, shiny, white, translucent
20-8	Anaerobic	round, convex, shiny, white, opaque
24-8	Anaerobic	round, convex, shiny, white, opaque
30-a-8	Anaerobic	round, flat, lumpy, smooth, white, translucent
30-b-8	Anaerobic	round, dome-shaped, smooth, dirty-white, translucent
31-8	Anaerobic	round, convex, shiny, white, opaque
33-a-8	Anaerobic	round, convex, shiny, white, translucent
33-b-8	Anaerobic	round, convex, shiny, white, variable size
37-7	Anaerobic	irregularly round, flat, shiny, white, translucent
39-7	Anaerobic	small, round convex, shiny, white
42-b-7	Anaerobic	small, round, convex, shiny, white, translucent
43-a-7	Anaerobic	round, quite convex, shiny, yellowish-white, opaque
44-a-7	Anaerobic	very small, round, convex, shiny
47-7	Anaerobic	round, convex, shiny, white, opaque

Discussion

The term Döderlein's bacillus is a name applied to the vaginal bacillus. In the current nomenclature this organism is classified as *Lactobacillus acidophilus*. For some time it has been believed that the Döderlein bacillus was not one specific type of organism. The preceding experimental results support this proposition.

In 2 cases, it was possible to isolate 2 strains of lactobacilli from a single patient. The resulting strains are 30A and B and 33A and B. As reference to the accompanying tables will show, strains 30A and 30B resemble each other morphologically with respect to colonial morphology and microscopic morphology. In addition to this, the similarity of their carbohydrate fermentation reactions leads one to believe that they are in reality members of the same strain. Strains 33A and 33B, on the other hand, exhibit marked differences in microscopic morphology, as well as in carbohydrate fermentation reactions. This has been interpreted as evidence that these are separate strains and lend further

evidence that the Döderlein bacillus is, in reality, a heterogenous group of organisms.

In most instances it is extremely difficult to classify the Gram-positive non-sporeing rods found in the vagina. They appear as large Gram-positive rods with the typical morphologic appearance of lactobacilli, but they also appear

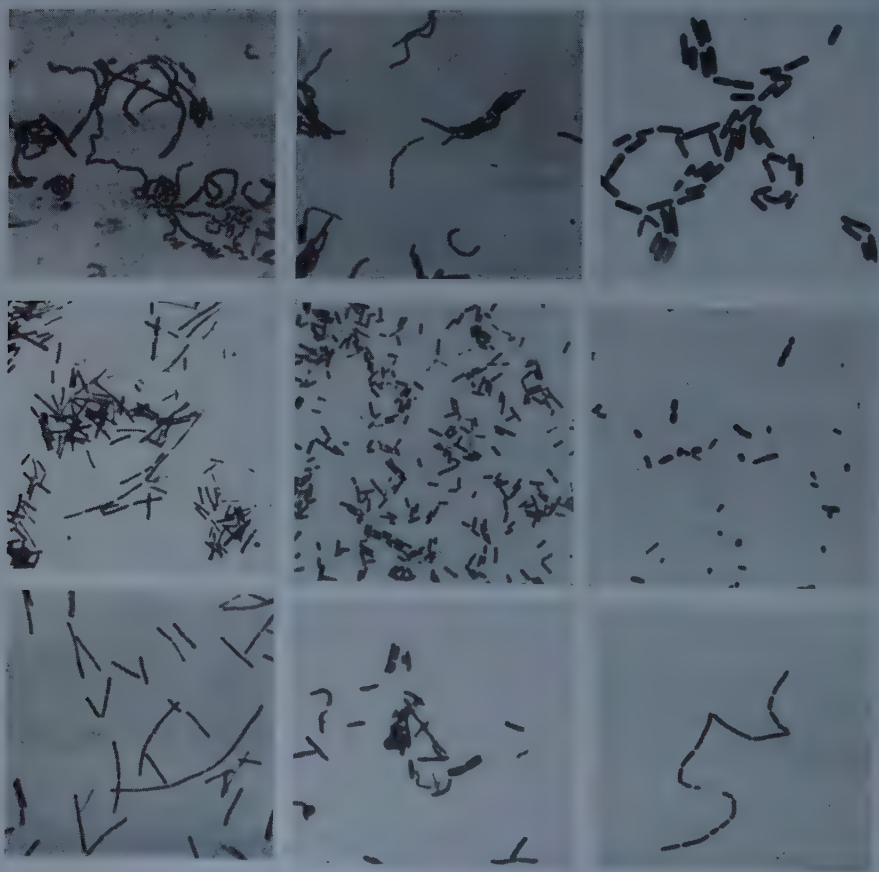


FIGURE 1. Photomicrographs of lactobacilli. (Upper left) Strain No. 73; (upper center) Strain No. 9; (upper right) Strain No. 20; (middle left) Strain No. 9224; (middle center) Strain No. 4962; (middle right) Strain No. 6; (lower left) Strain No. 17; (lower center) Strain No. 33A; and (lower right) Strain No. 33B.

in bizarre pleomorphic forms. In a previous paper, Hunter and Long,⁷ place these organisms in the genus *Lactobacillus*. Those organisms that met the criteria for this classification included the majority of the Gram-positive rods found in the vagina. There is no doubt that some corynebacteria or diphtheroids may have been included in this group. However, due to the present standards of classification of these two groups of organisms, it is practically impossible to separate them on a morphologic and cultural basis alone. This belief is in agreement with Wilson and Miles,⁸ who state that classification of

this group of organisms is unsatisfactory, partly because of some difference of opinion as to which organisms should be included in the lactobacillus group, and partly by reason of the absence of suitable criteria to serve as a basis of subdivision. Morphologic and colonial appearances vary considerably, and metabolic properties and fermentation reactions tend to show continuous, as opposed to discontinuous, variation; therefore, it is difficult to do more than select modal points around which strains can be grouped.

TABLE 5
COMPARISON OF CARBOHYDRATE FERMENTATION REACTIONS

Strain number	Incubation period	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Lactose	Sucrose	Maltose	Trehalose	Raffinose	Starch	Dextrin	Glycogen	Mannitol	Sorbitol	Dulcitol	Inositol	Salicin
	<i>hours</i>																			
761	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
77	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
73	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
44	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
79	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
83	24	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
483	24	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	48	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
78	24	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
	48	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
60	24	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	48	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
9857*	24	+	-	-	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-
	48	+	-	-	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	+
9224*	24	-	-	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	+	-
	48	-	-	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	+	-
4962*	24	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-
	48	-	-	-	+	+	+	+	-	+	+	-	-	+	-	-	-	-	-	-

* Strains from the American Type Culture Collection.

It may also be well to add that, although great strides have been made in formulating adequate media for the isolation and maintenance of lactobacilli, this problem is still far from overcome. With the media used in this study, we failed to isolate specimens of lactobacilli in a significant percentage of cases, even when the direct smears showed organisms of the typical morphology to be in abundance. This problem severely hampers efforts to classify these organisms.

It is generally understood that the differentiation of any species of bacteria cannot be determined by any one factor, such as fermentation reaction, but all factors affecting the organisms must be considered. This is well illustrated by the variable fermentation and other characteristics of the lactobacilli. At the present, there is no generally accepted method for truly classifying this group of organisms.

TABLE 6
COMPARISON OF CARBOHYDRATE FERMENTATION REACTIONS

Strain number	Incubation period	Arabinose	Rhamnose	Xylose	Glucose	Galactose	Maltose	Trehalose	Raffinose	Starch	Dextrin	Mannitol	Sorbitol	Dulcitol	Inositol	Salicin	Saccharose
5-8	24 hr.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	48 hr.	—	—	—	—	—	+	—	—	+	—	—	—	—	—	—	—
	9 days	—	—	—	—	+	+	+	—	+	+	—	—	—	—	—	—
6-8	24 hr.	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—
	48 hr.	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—
	9 days	—	—	—	+	+	+	+	—	+	+	—	—	—	—	+	—
15-8	24 hr.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	48 hr.	—	—	—	—	—	+	—	+	—	—	—	—	—	—	—	—
	9 days	±	±	+	+	—	+	—	+	—	—	—	—	—	—	—	±
17-b-8	24 hr.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
	48 hr.	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	+
	9 days	—	—	—	+	+	+	—	+	—	+	—	—	—	—	—	+
30-a-8	24 hr.	—	+	—	+	+	—	—	—	—	+	—	—	—	+	+	—
	48 hr.	—	+	—	+	+	+	+	—	+	+	—	—	—	+	+	+
	9 days	—	+	—	+	+	+	+	+	+	+	—	—	—	+	+	+
30-b-8	24 hr.	—	+	—	+	+	+	—	—	+	+	—	—	—	—	+	—
	48 hr.	—	+	—	+	+	+	+	+	+	+	—	±	—	—	+	—
	9 days	—	+	—	+	+	+	+	+	+	+	—	±	+	+	+	—
31-8	24 hr.	—	+	—	+	—	—	+	+	+	+	—	—	—	+	+	+
	48 hr.	—	+	—	+	+	+	+	+	+	+	—	—	—	+	+	+
	9 days	—	+	—	+	+	+	+	+	+	+	—	—	—	+	+	+
33-a-8	24 hr.	—	—	+	—	—	—	—	+	+	+	—	—	—	—	—	—
	48 hr.	—	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—
	9 days	—	—	+	+	+	+	+	+	+	+	—	—	—	—	+	+
33-b-8	24 hr.	—	+	—	+	+	+	+	—	+	+	—	—	—	+	+	—
	48 hr.	—	+	—	+	+	+	+	—	+	+	—	—	±	+	+	+
	9 days	—	+	—	+	+	+	+	—	+	+	—	—	+	+	+	+
37-7	24 hr.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	48 hr.	—	—	—	—	—	+	—	+	—	—	—	—	—	—	—	+
	9 days	—	—	—	+	+	+	—	+	+	+	—	—	—	—	—	+
42-b-7	24 hr.	—	—	+	+	—	—	—	+	+	+	—	—	—	—	—	+
	48 hr.	—	—	+	+	+	+	—	+	+	+	—	—	—	—	—	+
	9 days	—	—	+	+	+	+	—	+	+	+	—	—	—	—	—	+
43-a-7	24 hr.	—	—	—	+	+	+	—	+	+	+	—	—	—	—	—	+
	48 hr.	—	—	—	+	+	+	—	+	+	+	—	—	—	—	—	+
	9 days	—	—	—	+	+	+	—	+	+	+	—	—	—	—	—	+
1-8	24 hr.	—	—	+	+	—	—	—	—	+	+	—	—		—	+	—
	48 hr.	—	—	+	+	—	+	+	—	+	+	—	—		—	+	—
	9 days	—	—	+	+	—	+	+	+	+	+	—	—		—	+	±

At the present time it appears foolhardy to assign any species designation to most of the organisms shown in this study. At best, they can be included only in a general classification. This study only helps to show that the Döderlein bacillus is not one specific organism, but a group of organisms. Whether it is important to assign species rank to these organisms is doubtful.

TABLE 7
REACTIONS IN MILK

Strain number	Days of incubation										Final pH
	1	2	3	4	5	6	7	8	9	10	
9224*	—	—	—	AC	AC	AC	AC	AC	AC	AC	5.2
9857*	—	—	—	AC	AC	AC	AC	AC	AC	AC	4.6
4962*	—	—	—	AC	AC	AC	AC	AC	AC	AC	4.7
83	—	—	—	—	—	—	AC	AC	AC	AC	4.7
79	—	—	—	—	—	—	—	—	—	—	6.3
761	—	—	—	—	—	—	—	—	—	—	5.5
483	—	—	—	—	—	—	—	—	—	—	5.3

* Strains from American Type Culture Collection.

Control pH, 6.3.

Key: A, acid; C, coagulation.

TABLE 8
REACTIONS IN MILK

Strain number	Days of incubation							Final pH
	1	2	3	5	7	9	10	
5-8	—	—	A	A	A	AC	AC	5.2
6-8	—	—	—	A	A	A	AC	5.1
15-8	—	—	—	—	—	—	—	6.05
17-b-8	—	—	—	—	—	—	—	6.07
30-a-8	—	A	A	A	A	AC	AC	5.13
30-b-8	—	—	—	A	AC	AC	AC	5.4
31-8	—	—	—	A	A	A	A	5.65
33-a-8	—	—	—	—	—	—	—	5.75
33-b-8	—	A	A	A	AC	AC	AC	4.50
37-7	—	—	—	—	—	—	—	6.0
42-b-7	—	—	—	—	—	—	—	5.83
43-a-7	—	—	—	A	A	A	A	5.6
1-8	—	—	—	A	A	A	A	5.5

Control pH, 6.15.

Key: A, acid; C, coagulation.

Conclusions

The morphologic appearances and cultural reactions for 22 strains of Döderlein's bacillus are shown and compared with American Type Culture Collection strains of *Lactobacillus acidophilus* (Döderlein's bacillus), *L. bulgaricus*, and *L. acidophilus* (bifidus).

It is shown that the Döderlein bacillus is not one specific organism.

Due to lack of suitable criteria to serve as a basis of subdivision, these organisms can be included only in a general group.

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THE STRUCTURE AND BACTERIOLOGY OF THE PREMENARCHAL VAGINA

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The changes that occur in the vagina between birth and the menarche are of interest because of their relation to a number of conditions affecting the child's health. This paper does not consider in depth those changes as regards detailed anatomy and microbiology, but does consider the morphology and bacteriology of the premenarchal vagina from the broader aspect of their relationship to the clinical problems that confront the clinician interested in pediatric gynecologic problems.

The descriptive material presented herein has been drawn in part from investigative reports that have appeared in the literature during the past 4 decades¹⁻³⁴ and in part from my own examination of 552 premenarchal patients. The latter group includes 21 girls who showed signs of sexual precocity, 14 who were either true hermaphrodites or pseudohermaphrodites, 12 who suffered from gonadal dysgenesis, 6 who had congenital anomalies of the vagina not secondary to congenital adrenocortical hyperplasia, 51 who had labial adhesions that were not congenital, and 5 who had lichen sclerosus et atrophicus. In addition, there was a number of single cases in which study of the clinical problems has been helpful in preparing this report.

The methods of study in appropriate cases included the following: vaginoscopy, digital-rectal palpation, vaginal cytoscsmears stained by the method of Papanicolaou or Shorr, vaginal cultures prepared and examined by standard laboratory techniques, suspension of vaginal fluid examined for fungi, trichomonads, and parasite ova, paraffin swabs for the collection of perineal parasite ova, and roentgenologic vaginograms and examination under anesthesia. The diameter of the hymenal orifice and the length of the vagina have been determined in children at the several periods of development by the use of rods of known length and diameter. The reaction of the vaginal fluid has been determined by the application of nitrazine paper inserted through a vaginoscope.

The alterations that occur in the vagina and also in the other genitalia lend themselves to the following four divisions of the life span between birth and the menarche: (1) the neonatal period, (2) the period of early childhood, (3) the period of late childhood, and (4) the immediate premenarchal period. Each of these periods is marked by characteristics that respond to hormonal stimulation. The appearance of these characteristics is usually correlated with somatic secondary sexual and chronological growth. They are not sharply demarcated one from the other; rather, one period merges into the next.

Structure

Neonatal period. For a little while the vagina of the newborn infant retains the morphology it possessed in late fetal life. When the infant is separated

from its intrauterine environment, the genitalia, unlike some of the other organ systems, do not manifest sudden physiological alterations.

The turgidity and succulence of the vulvovaginal mucosa that are present at birth and during the neonatal period are the result of antenatal maternal hormonal stimulation. The hymen forms a thick, pouting, inverted cone having one central orifice measuring approximately 0.4 cm. in diameter. The vaginal mucosa, when examined vaginoscopically, is pale pink and succulent. It is folded into high ridges and deeper crypts by rugae circularly arranged in the deeper vagina. The depth of the vagina is approximately 4 cm. in the newborn infant; it is a narrow, elongated tube. The ruffled epithelium covering the cervix is continuous with that of the vagina. The external *os* is not distinctly formed, and the boundaries of the external cervix are indefinite at birth. So-called congenital erosions have been described, but they are rarely seen and are probably the result of imperfect antenatal differentiation of the paramesonephric and sinal elements that form the cervical and vaginal epithelium.

The neonatal period lasts for approximately 2 weeks. During that time the effect of the maternal hormones gradually wanes. Histologically, the vaginal mucosa at birth consists of 20 to 40 cell layers. The outer layers are cornified, and there is active desquamation. Glycogen is present in generous amounts. Cytosmears are very similar to those obtained from postmenarchal adolescents. The vaginal canal contains a white, turgid material consisting almost entirely of desquamated epithelial cells. At birth the vaginal reaction ranges between a *pH* of 5.5 and 7.0. After 24 hours the *pH* is lowered to between 5.0 and 4.0, due to the formation of lactic acid that follows the appearance of lactobacilli in the vaginal flora. The reaction is acid for a few days and then gradually becomes neutral and alkaline as the effects of the mother's hormones recede. The lactobacilli are replaced by other organisms, and lactic acid disappears from the vaginal fluid.

Period of early childhood. The period of early childhood lasts from the time the stimulation from the mother's hormones is withdrawn until the child begins to produce her own endogenous estrogen. During this period the hymen is thin and flat with a delicate edge. The central orifice is unchanged in size. The inelastic vaginal vault forms a long narrow cavity. The vagina grows only 0.5 cm. to 1.0 cm. in depth. The vulvovaginal mucosa is thin and hyperemic. The vaginal epithelium varies from 2 to 8 cells in thickness and contains little or no glycogen. Cytosmears reveal small round cells with darkly stained cytoplasm and large nuclei; there is no cornification. The vaginal fluid is scant and has a *pH* ranging from 6.5 to 7.5. The poorly formed labia majora and attenuated labia minora give the introitus very little protection. The cervix is small, often hidden, and appears as a flat elevation at the apex of the vagina. The external *os* is diminutive. There is no cervical secretion. The major vestibular glands are inactive.

Period of late childhood. The period of late childhood is one of awakening ovarian, hormonal, and secondary sexual development. Major alterations in the growth of the vaginal structures do not keep pace with those of the breast and pudendal hair. However, careful observation of lesser changes in vaginal morphology and cytology reveal that the vaginal response to the initiation of

ovarian secretory function is more definite than are changes in the breasts and pudendal hair.

Changes in the vagina, marking its first response to ovarian activity, are evident in the increasing size of the hymenal orifice, the lengthening of the vaginal canal, the beginning cornification of the vaginal epithelium noted in vaginal cytosemears, and alterations in the size and shape of the cervix. At first, the gross appearance of the vulvar and vaginal epithelium may not appear to be changed. The mucosa of the vulva and distal one third of the vagina remain thin and hyperemic. In many instances, however, vaginoscopic visualization of the upper one third of the vagina will show a little less hyperemia of the epithelium adjacent to the cervix.

During early childhood the hymenal orifice measures between 0.1 and 0.7 cm., with an average of 0.4 cm., in diameter. During the period of late childhood, long before the vulvovaginal mucosa reveals any definite amount of estrogen response, the introitus increases several millimeters in diameter. The hymenal orifice will measure 0.7 cm. or more in diameter by the time the average girl shows a gross vulvovaginal estrogen response. The introitus of most girls who are near the menarche will admit an instrument 1 cm. in diameter.

Lengthening of the vagina begins with the first sign of isosexual development and continues through the period of late childhood. During early childhood the average depth of the vagina is from 4.5 to 5.5 cm. By the time there are evidences of estrogen response in the vulvovaginal mucosa, the depth of the vagina is from 7.0 to 8.5 cm. with an average depth of 8.0 cm. Near the menarche, the vagina has a depth of 10.5 to 11.5 cm.

Changes in vaginal cytology also begin in late childhood. The epithelial cells become larger, the nuclei are of medium size, and the protoplasm stains more lightly. Cornification of 5 to 15 per cent of the cells generally is present before there are other signs of sexual development and before the mucosa of the vulva and distal two thirds of the vagina have shown any gross signs of ovarian response. The vaginal fluid continues to be scant in amount and to have a pH of 6.5 to 7.5.

As these changes are taking place, the vaginal portion of the cervix becomes larger. It gradually acquires a flat, buttonlike shape or assumes a conical form. The cervical glands are inactive, and the canal is visualized with difficulty.

Immediate premenarchal period. The immediate premenarchal period lasts for 3 to 18 months. During this time the vulvar and vaginal mucosas lose their thin, glazed, hyperemic appearance and become pink, thick, and soft. The hymen again becomes thicker and more cufflike. There is an increased amount of vaginal fluid, which has a pH of 5.5 to 4.5 and consists largely of desquamated adult-type squamous cells, most of which are cornified. The vaginal epithelium is many cell layers thick and is richly laden with glycogen. The cervix acquires a mushroom shape. The size of the hymenal orifice and the depth of the vagina are not appreciably larger, but the increased succulence and elasticity of the vulvovaginal tissues make for greater flexibility. The upper vagina is more distensible, but deep fornices are not formed. The cervical and major vestibular glands become secretory.

The menarche marks the sudden change in the physiological activity of the genitalia and often in the young woman's psychosexual attitude. It does not, however, mark a critical change in the gross structure of her vagina.

Abnormalities of structure. Deviations from normal growth patterns and abnormalities of vaginal structure are encountered in cases of sexual precocity, congenital adrenocortical hyperplasia, gonadal dysgenesis, other types of hermaphroditism, pituitary disturbances, and congenital anomalies limited to the vagina. Signs of a hormonal response in the vaginal tissues in early childhood and an absence of such a response, which should be present, when the child is older are specific findings in the study of endocrinopathic problems associated with precocious or delayed growth of the genitalia. Adherent labia minora are postinflammatory phenomena; they are not congenital anomalies. They are frequently mistaken for vaginal agenesis or an imperforate hymen.

Differences in the size of the hymenal orifice in different children seem to be congenital variations and represent the most frequent variable in vaginal structure. They are without significance except insofar as they affect the ease with which vaginostomy can be performed.

The vagina retains the characteristics of early childhood in patients suffering from gonadal dysgenesis, hypopituitarism, or premenarchal ovarian ablations. Its gross configuration remains unaltered.

Congenital adrenocortical hyperplasia and other types of hermaphroditism produce distortions of the vagina of varying degrees and character. It is interesting to note that the most frequent abnormalities of the vagina found in patients suffering from adrenocortical hyperplasia involve the vulva and lower one third of the vagina. Vaginograms will reveal a well-developed vaginal vault and a small cervix. Below them, a narrowed, almost obliterated distal canal forms a shallow urogenital sinus.

Bacteriology

Obtaining a satisfactory culture from a child's vagina is not a simple procedure. The premenarchal vestibule is much deeper and the introitus much smaller in proportion to those of the adult; furthermore, the youthful patient is sometimes recalcitrant. If the material is not collected with particular care it will inevitably be contaminated by the vulvovestibular secretions. Material collected on cotton applicators inserted into the hymenal orifice, fluid obtained at the introitus by intrarectal pressure on the posterior vaginal wall with the examiner's fingers, and secretions from the vaginal orifice for vaginal cultures do not give a true picture of the vaginal bacterial population. Cultures should be obtained by the introduction of a small wire loop through a sterile vaginoscope inserted through the hymenal orifice.

When the child is suffering from a nonspecific vaginitis the areas within the vagina from which the material is obtained also seem to be a factor in determining the predominant type of organism. In many instances a nonspecific inflammation involves the vulva and lower one third of the vagina, but does not affect the upper two thirds of the vaginal mucosa or the cervix.

The results of a number of studies on the bacteriology of the vagina in childhood have been reported. The findings which have been reviewed by Lang

agree for the most part with the observations of Kotcher *et al.*¹⁸ that the healthy vaginas of normal girls contain a variety of organisms.

My own observations to date are similar in most respects to those of other reporters. Although the work is still in progress, a current report on it will supplement their findings. A final report will be published when the investigation is completed.

Vaginal cultures have been taken from 434 girls in the Pediatric Gynecologic Clinic at Children's Memorial Hospital, Chicago, Ill., from hospitalized clinic patients and from private patients at the hospital and in the office. The patients have been divided into those who are in the periods of early and late childhood and those who are in the immediate premenarchal period. The division is based on clinical evidence of beginning sexual maturation or on estrogen response as seen in vaginal cytosemears. The two major groups mentioned above have been subdivided into those with or without signs or symptoms of vulvovaginal inflammation. The cultures were taken from the upper vagina through a vaginoscope, as previously described.

There were 237 girls who had no signs or symptoms of vulvovaginitis. Of these, 101 were in early and late childhood. In this group there were 136 girls who gave evidence of ovarian activity. There were 21 patients who were isosexually precocious. There were 197 girls who had vulvovaginal inflammation, discharge, or both. Of these, 143 were in early or late childhood, and 51 had signs of ovarian activity.

Neonatal period. The lower genital tract of the infant is free of bacteria at birth. Twenty-four hours later the vagina is invaded by nonhemolytic coagulase-negative staphylococci, diphtheroids, and enterococci. Vaginal cultures taken four days postnatally show a heavy growth of lactobacilli and relatively few other organisms. A week later the lactobacilli have disappeared, and the vaginal flora again consists of a variety of organisms.

Early and late childhood. The bacteriology of the healthy vagina does not change appreciably during the time between the end of the neonatal period and the onset of the immediate premenarchal period. Kotcher *et al.*¹⁸ have recently studied the vaginal microbiology in 57 children with vaginitis and in 25 controlled patients. These investigators report that a large number of species of various bacteria was isolated from the vaginas of children having vaginitis and from normal girls in a control group in approximately equal percentages.

Organisms not discovered in their control group were found in the patients having vaginitis; these included *Trichomonas vaginalis*, *Candida albicans*, and any of the *Neisseria*. The concept is generally accepted that lactobacilli do not reappear as the predominant flora in the vagina until the immediate premenarchal period.

Vaginal cultures that I have obtained from girls in early and late childhood without vulvovaginitis have revealed the presence of a wide variety of various species of bacteria. For the most part, these were diphtheroids, nonhemolytic coagulase-negative strains of *Micrococcus pyogenes* var. *albus*, and nonhemolytic *Streptococcus*. Bacteria identified as *Escherichia coli* were isolated from vaginal cultures in 16 per cent of the girls in this group. No *T. vaginalis*, *C. albicans*, or *Neisseria* were identified. At the start of the study, cultures were

obtained by sterile cotton applicators passed through the hymenal orifice. They almost invariably contained *E. coli*.

Vaginal cultures from girls who gave evidence of ovarian function and who did not have vulvovaginitis varied from those in the preceding group. There was a reduction in the incidence (9 per cent) of bacteria identified as *E. coli* and the preponderance of nonhemolytic coagulase negative strains of *Micrococcus pyogenes* var. *albus*. None of the girls in this group harbored *T. vaginalis*, *C. albicans*, or *Neisseria*. It is accepted that the rising estrogen level of the immediate premenarchal period is associated with an increase in the re-establishment of lactobacilli in the vagina, with the appearance of lactic acid in the vaginal fluid and with an acid reaction.

Vaginal Bacteriology and Vaginitis

Vaginal cultures from 143 girls in early and late childhood suffering from vaginitis revealed a significant difference in the vaginal flora when compared with cultures from girls in the same period who had neither vaginal discharge nor inflammation. Fungi were found in the vaginal discharge in 7 cases, and *Neisseria gonorrhoeae* in 2 cases. *E. coli* were isolated in 149 (77 per cent) of the cultures. In addition, a variety of other species of bacteria was identified. Although they were not found in cultures from girls who did not have vaginitis, they nevertheless occurred so sporadically that their relation to the inflammation with which they were associated could not be assessed.

Vaginal cultures also were taken from 51 girls who had signs of ovarian activity. In this group, fungi were found in 10 cases, *T. vaginalis* in 5 cases, and *N. gonorrhoeae* in 1 case. *E. coli* were isolated in 16 cases, or approximately 31 per cent of the cultures from this group.

The inflammation involved the entire vaginal mucosa and was associated with a discharge in each instance in which the vaginitis was associated with vaginal fungi, *T. vaginalis*, or *N. gonorrhoeae*.

It will be noted that mycotic infections were not limited to either age group, but that *T. vaginalis* was found only in older girls. Mycotic infections were associated with a history of previous antibiotic therapy in 14 of 17 cases. In no instance was there evidence of the coital transmission of *T. vaginalis*.

The ova of *Enterobius vermicularis* were found in the vaginal discharge of 2 patients. Intestinal infestations of pinworms were found in 10 girls who had vaginal discharge and vaginal inflammation. I believe that the relation between intestinal parasitism and vaginal discharge and inflammation is more intimate than this figure indicated. It is interesting to note that in each instance intractable vaginitis was associated with the presence of pinworms, and that *E. coli* were found as the predominant organisms in vaginal cultures in these cases.

Discussion

It is not easy to arrive at a chronologic base line in the several periods of structural and bacteriological changes in a healthy premenarchal vagina because they are related to a chronologic variable, namely, the appearance of the menarche. If we use the generally accepted concept that the first menstrual period may occur between the ages of 10 years and 16 years, then the

periods of early and late childhood may be limited to 9 years or less, or may extend to 14 years or 15 years.

The neonatal period is not subject to much variation. The infant almost invariably will not retain the effect of the maternal hormones for longer than 3 or 4 weeks, and usually less. If 13 years of age is selected as the time of the menarche, the period of early childhood will most often merge into the period of late childhood when the child is 7 or 8 years old. The immediate premenarchal period usually begins between 6 to 18 months prior to the menarche.

Knowledge relative to the structure of the premenarchal vagina is a necessary prerequisite to the gynecologic examination of the child. The small size of the hymenal orifice, the relatively long vaginal canal, and its inelastic form are factors that make vaginal inspection during childhood a delicate and, at times, rather difficult task.

Vaginal cytology has proved to be the most delicate of the several signs signaling the onset of ovarian function. There are little girls who have enlargements of the breasts and pudendal and axillary hair growth several years before they have their first menstrual period. In such cases, particularly, the vaginal smear has proved a definitive means of determining whether such phenomena are specific indicators of sexual precocity or are indicative of increased end-organ sensitivity. Conversely, there are rare instances of idiopathic clitoral hypertrophy in late adolescence or combined virilism and feminization in early childhood in which the vaginal cytosmear becomes a helpful and precise tool for estimating qualitatively the endogenous production of estrogen or estrogenlike substances.

It has been customary to classify vaginitis in premenarchal girls as either gonococcal or nonspecific. Kotcher *et al.*¹⁸ have suggested that the latter category should be classified as nongonococcal vaginitis. Only very rarely is infection in the lower genital tract of premenarchal girls limited to the vagina; almost inevitable there is an associated vulvitis. The presence of fungi and trichomonads in association with vaginal inflammation has been demonstrated. Vulvovaginal inflammations with the exanthemas diphtheritic and pneumococcic vaginitis have been reported. Lang²⁰ has referred to the association of vaginitis to upper respiratory infections.

The incidence of proved gonococcic vulvovaginitis in the premenarchal children whom I have examined is low when compared with the group reported by Kotcher *et al.*¹⁸ The latter reported that *N. gonorrhoeae* were identified in 21 per cent of their patients. The difference between the two series can not be explained; it may reveal differences in socioeconomic levels, in patient groups, or in preadmission care. Many of the children I examined have been referred from other clinics.

The etiological agent in many cases of nongonorrheal vulvovaginitis has not been incontrovertibly identified in either the premenarchal or adult female. Gardner and Dukes have presented strong evidence implicating *H. vaginalis* as the specific causative organ. However, Kotcher *et al.* did not isolate *H. vaginalis* from any of the 82 girls in whom they obtained cultures. The latter authors have suggested that some viral agent or agents such as the Coxsackie virus or possibly some other as yet unrecognized virus may be the etiological entity that has so far escaped detection.

The frequency with which bacteria that normally inhabit the gastrointestinal tract are found in association with nongonorrheal vulvovaginitis in children strongly suggests to me that, whatever agent it is, it seems highly probable in most instances that it reaches the vagina by way of fecal contamination.

The occurrence of nongonorrheal vaginitis secondary to foreign bodies, upper respiratory infections, and infections of the urinary tract merits mention. Postinflammatory adhesions of the labia minora are the result of vulvitis. These adhesions are a factor in causing vaginal inflammation when they deflect part of the urinary stream into the vagina. The cervix of the premenarchal child is not an etiological factor in vaginitis and rarely participates in the inflammatory reaction. Erosions of the cervix were found in only 2 of the 552 premenarchal patients whom I examined, and both of these girls were in the immediate premenarchal period.

Vaginal discharge is a symptom of inflammation and infection in girls in early and late childhood. The neonatal period, however, is characterized by the

TABLE 1
ETIOLOGICAL CLASSIFICATION OF VULVOVAGINITIS

1. Gonococcic vulvovaginitis
2. Nongonococcic vulvovaginitis
a. Nonspecific
(1) Etiology undetermined
(2) Secondary to:
(a) Upper respiratory infections, parasites, foreign bodies, urinary tract infections
(b) Trichomoniasis
(c) Mycotic
(d) Pneumococcic
(e) Exanthemata, etc.

presence of a curded white material in the vagina; it consists of desquamated epithelial cells. The immediate premenarchal period is also characterized by an increase in vaginal fluid that frequently causes the mother of the child to believe that the latter has an abnormal discharge. Examination will often reveal unduly succulent, but otherwise normal, vaginal epithelium and a copious amount of fluid in the vagina consisting of desquamated vaginal cells and cervical secretions.

In view of the above, the term vaginitis as applied to the premenarchal child should be replaced in most instances by the term vulvovaginitis. The latter should be classified etiologically as shown in TABLE 1.

Summary

This presentation includes a review and summary of certain work in progress related to the structure and bacteriology of the premenarchal vagina.

The neonatal period, the periods of early and late childhood, and the immediate premenarchal period are marked by distinct differences in vaginal structure and microbiology. These periods merge into one another, but the changes that occur during them are sufficiently marked to serve as criteria of genital growth.

The changes that occur in the vaginal epithelium, as indicated by vaginal

cytoscarns, are delicate indicators of ovarian endocrine function and frequently are more sensitive signals of gonadal awakening than are changes in other estrogen-sensitive end organs.

Cultures from the vaginas of healthy children, when not contaminated by vulvar material, are relatively free of pathogenic organisms.

The etiology of nongonorrheal and nonspecific vulvovaginitis is not known, but in view of the high incidence of coliform bacteria in vaginal cultures from girls having vulvovaginitis or inflammation and discharge, it is suggested that the etiological agent probably is transmitted from the gastrointestinal tract to the vagina by fecal contamination.

Mycotic infections of the vagina are not limited to any age group, whereas *T. vaginalis* was found only in children in the immediate premenarchal period.

An etiological classification of vaginal infections in childhood is suggested.

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ABDOMINOVAGINAL ELECTROPOTENTIAL DIFFERENCES IN THE MENSTRUAL CYCLE*

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The generation of minute electropotentials is a property of unicellular and multicellular organisms that seems to reflect their over-all metabolic activity, as well as the exchange of hydrogen ions and electrolytes with their environment. In the human, two areas with acid secretory epithelial surfaces have been found to generate significant potentials: the gastric mucosa and the vaginal epithelium. It should be possible to capture and record changes in potential associated with physiological activity of the ovary, particularly ovulation, as reflected by the vaginal mucosa. Vaginal and cervical potentials have been known to exist for at least twenty-five years. Previous attempts to measure significant potential differences that might pinpoint the day of ovulation have been inconclusive. The observations presented here suggest that, by employing a revised technique that utilizes a simplified electrode and a recording millipotentiometer that does not draw current from its external circuit, there is a mid-cyclic alteration of potential that appears with dependable regularity and is not recorded in the menopausal individual.

The chief points wherein the present studies differ from those previously undertaken lie in the sensitivity of the recording instrument and the type of electrode employed. The highly sensitive recording instrument employed in these studies is a vacuum tube recording millipotentiometer of high resistance that measures the changes in potential differences between a glass electrode inserted in the vagina and a similar reference electrode on the anterior abdominal wall. The entire instrument is grounded to the patient's leg. The potential changes are transferred from the liquid electrode through calomel half cells to reach the recording instrument (FIGURE 1).

The recording instrument has a resistance of 5 megohms that prevents current flow through the external circuit. The withdrawal of current from the tissues would tend to decrease the potential. An internal calibration of 10 mv potential is used to standardize the recording. The sensitivity of the instrument and the stability of recording are such that, once the machine has been balanced, no further manipulations are necessary. In methods previously described, rapid manipulations of the potentiometer were necessary to anticipate deflection of the galvanometer. Once the instrument is properly balanced, it is possible to record for as long a period as may be necessary. In practice, the recordings are made during a span of 15 to 25 min. while the patient rests quietly in bed.

Glass rod electrodes filled with 0.9 per cent saline and plugged with cotton have replaced the silver chloride electrodes used by previous investigators. Such electrodes are easier to use and maintain and have materially reduced the

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number of artifacts that may arise from metal contacts, bodily movement, vibration, and skin irregularity. The recordings appear to be independent of the pH or protein content of the surfaces in contact with the electrodes, as

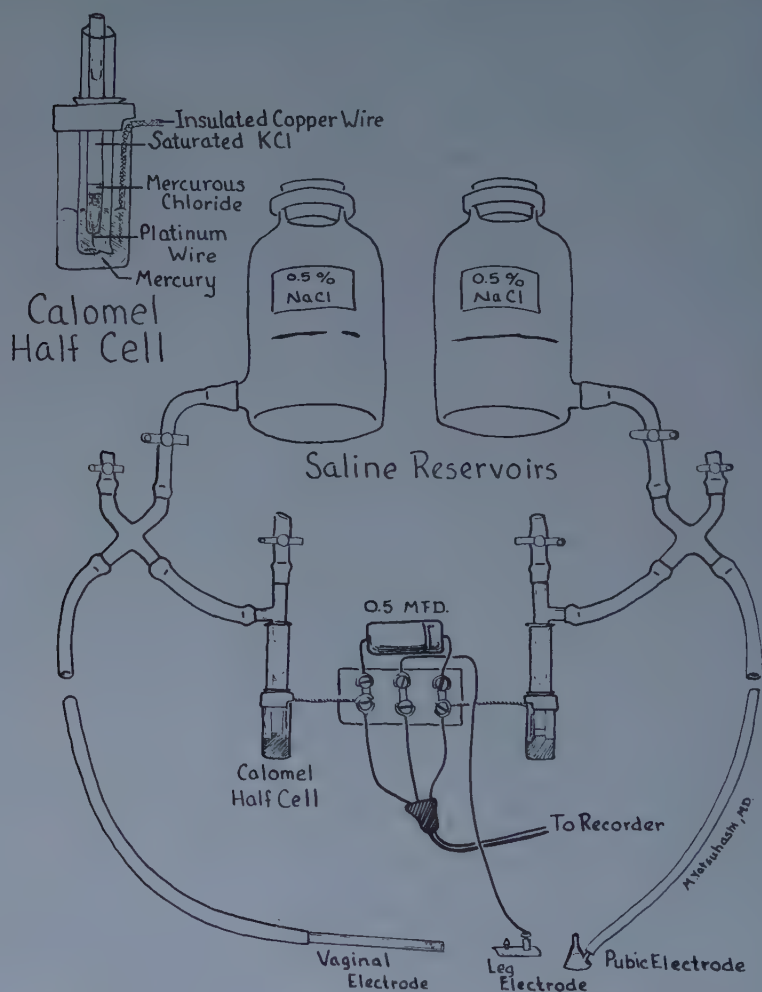


FIGURE 1. Schema of electrode system used in these experiments.

shown by studies carried out by Lemon and his associates,¹ who found this to be true in humans between a pH of 1 to 10.

Although the recordings seem to reflect metabolic activity of the vaginal epithelium, it is impossible at this stage of the investigation to declare with certainty what we are measuring, but it would appear that the method does provide a standard for recording ovarian activity and the fluctuation in ovarian hormone output.

Clinical Observations

The following generalizations are made on the basis of vaginal recordings of nearly 3000 tests:

The ages of the patients varied from 18 to 88 years. Physiologically, they have been grouped either as premenopausal, menopausal, or postmenopausal. The initial voltage varies, depending on the physiological group of the patient as well as the phase of the menstrual cycle. To determine the normal base line for each patient, readings were taken during the menses and for 7 days thereafter.

The interpretations of the readings are based upon the initial voltage, the final level of voltage obtained, and the length of time required to reach the final level. The slope of the curve determines the degree of cyclic activity.

There is a marked difference in the recordings of electrovaginal potential in normal premenopausal and postmenopausal women. Following menstruation, normal women show successive daily changes in their initial electro-potential differences and the type of curve recorded. The voltages are maintained in the negative range. The readings are reproducible from patient to patient and from the day of one cycle to the corresponding day of the next or later cycles.

No such cyclic variation is found in the postmenopausal woman. Although the voltages may appear in the negative or positive range, depending possibly on the amount of estrogenic activity present, the curve is flat. When the ovaries have been removed or irradiated, the flat curve appears in positive ranges of plus 40 to 60 mv (FIGURE 2).

Variations Noted in the Menstrual Cycle

The characteristics of the recorded curves as they appear in the different stages of the menstrual cycle are as follows:

Postmenstruation. No potential difference is noted shortly after menstruation, as shown by the flat curve. Voltages are maintained in negative ranges from minus 20 to minus 8 mv, with variations noted in the base-line level of each patient.

Preovulatory. Reaching what we believe is the time of ovulation in the mid-portion of the cycle, the curve rises sharply from minus 20 to plus 20 or 30 mv in the space of 10 to 15 min. There is a steep inclination to the curve.

Ovulation. In the middle portion of the cycle a marked change in the potential appears. The curve remains flat with recordings in the positive range (plus 12 to plus 22 mv) that may persist for 1 or 2 days. We believe that ovulation occurs at this time. This lack of functional activity corresponds to the same quiescent phase that Markee² noted in the ocular implants of endometrium.

Postovulatory. After 1 or 2 days the flat curve sustained in the positive range gives way to the same type of curve noted before the so-called ovulating point. The curve rises sharply in 10 to 15 min. from a negative potential to positive readings of 25 to 30 mv.

Premenstrual. The premenstrual curve is relatively flat, with readings in

the range of minus 4 to minus 10. As the period approaches, the slope of the curve tends to increase slightly.

The numerical values of the days of the cycle may vary, but the flat potential after a preovulatory rise is constant in all normal premenopausal cycles (FIGURE 3).

Vasomotor disturbances, minute variations in skin temperature, and humidity, and focal changes in pH have all been advanced as possible explanations of the potential variations occurring in the menstrual cycle. Temperature,

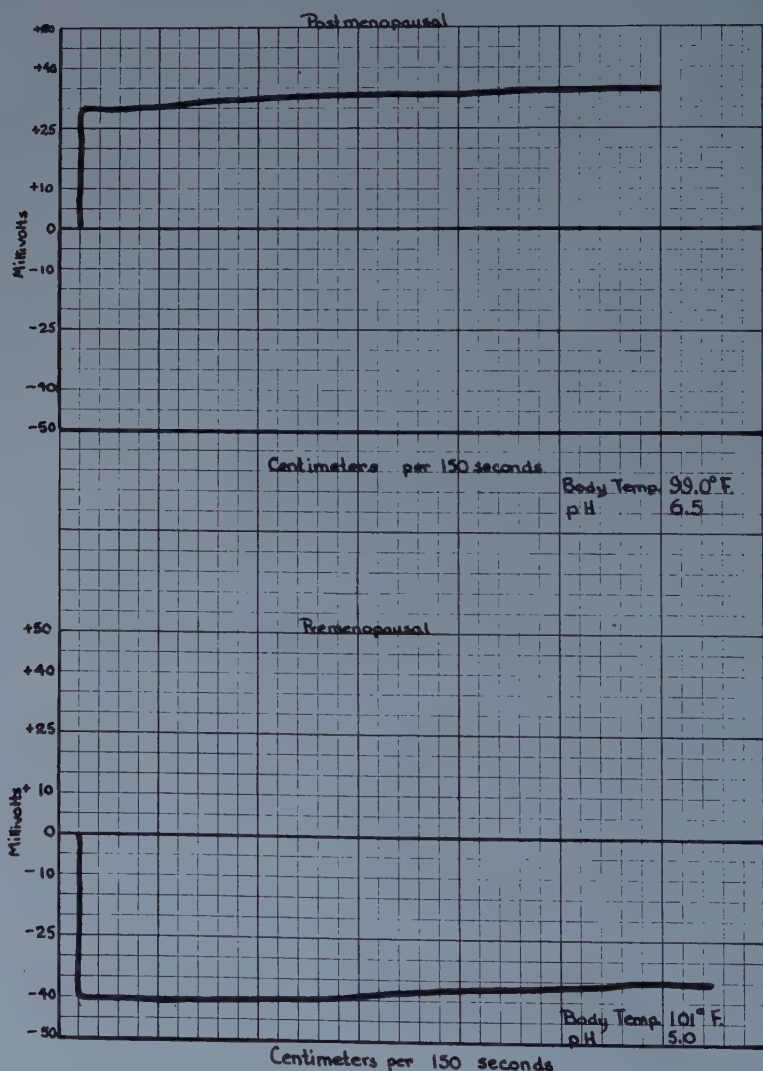


FIGURE 2. Comparison of voltages of a postmenopausal patient and of a premenopausal patient.

pH, and psychogenic factors may influence the potential but, in our opinion, do not entirely account for the repetitive and cyclic changes observed during the menstrual cycles associated with ovulation.

Clinical Application of the Method

Several disturbing factors have come from the observations made to date that may have widespread clinical significance.

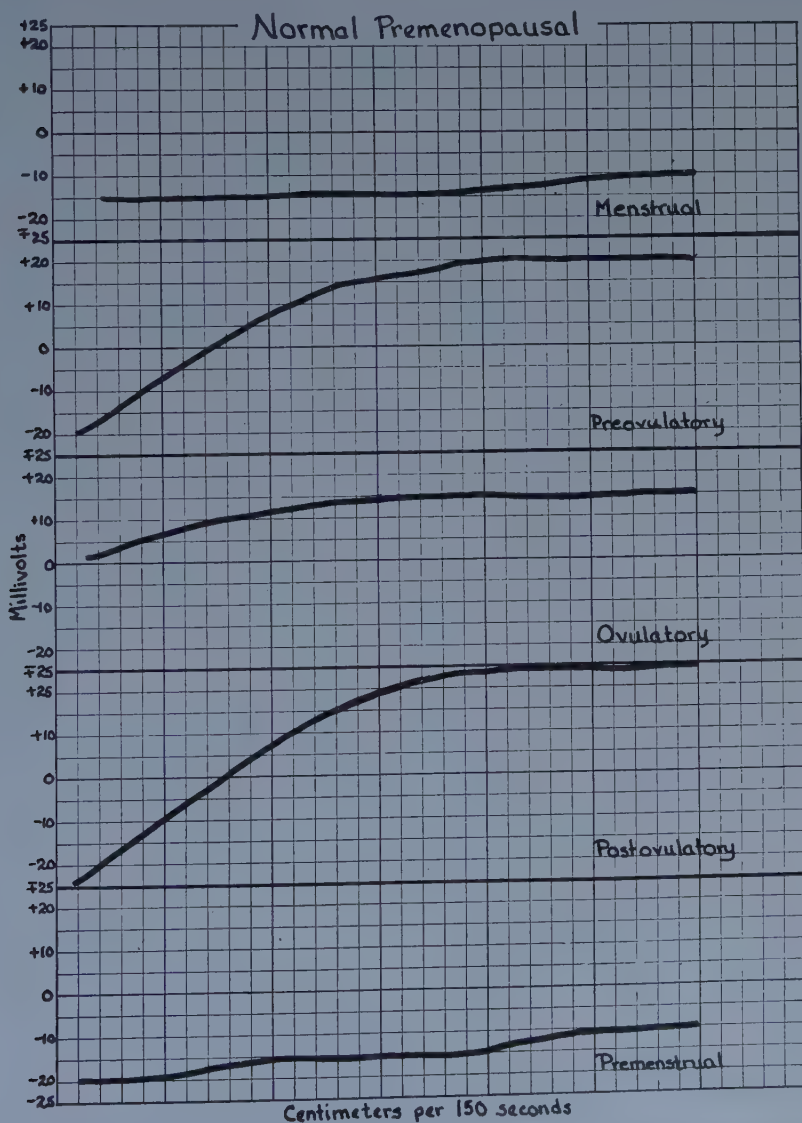


FIGURE 3. Typical cyclic curves of a normal menstrual cycle recorded at the labeled stage.

Dating of ovulation. The flat curve in the positive range noted in the central portion of the cycle varies in the time of its appearance, depending on the length of the cycle. In the girl who has a 28-day cycle the phenomenon is usually noted on the tenth day from menstruation. When the cycle is delayed (for example, 35 days), the same sort of recordings are observed on the fifteenth day. With foreshortened cycles the so-called ovulatory date appears on the sixth or seventh day.

It is interesting to note that the examination of pregnanediol tends to follow the same cyclic pattern noted in the electrovaginal recordings. During the 24- to 48-hour period when the curve remains flat and we assume that ovulation is taking place, the urinary pregnanediol disappears almost completely. The variations are not minute quantitative differences, but approach an all-or-none transition. The pregnanediol reappears as the vaginal recordings again show the sharp rise from the negative to the positive range.

If the observations are correct and the readings reflect ovulation, then either the patients are ovulating earlier than we have heretofore believed, or the progestational phase is in the range of 20 days and not 14 days, plus or minus 2.

This is a change in concept that is very difficult to justify. Little help can be obtained from the Fern test,³ basal body temperatures, changes in vaginal pH, glycogen staining of the vaginal smears, or endometrial biopsy. All are subject to great variation, and measure ovulation only after it has occurred. Much investigative work must be done. At present, we have only clinical observations to support the contention that the electrovaginal potentials record the date of ovulation.

Fertility. The information gathered from a study of the ovulatory pattern of normal women with different types of menstrual cycles has been applied to a group of women who presented problems in fertility. Only those patients who had been married for from 3 to 10 years and had tried faithfully to become pregnant for at least 2 years were studied. The majority had had a complete sterility investigation elsewhere and had been considered as severe infertility problems before we began our testing.

At the present time 51 patients have completed their studies. Twenty-seven have become pregnant simply when we pointed out the day on which we believed they were ovulating and asked them to concentrate on this date. Several had intercourse only on their supposed ovulatory dates. Others had had several unsuccessful artificial inseminations on dates later in their cycles. One patient had 6 artificial inseminations over a 10-year period performed on the sixteenth day, which had been selected because of the information produced by basal body temperature charts. She became pregnant at 37 years of age, on the tenth day of her cycle, which was selected because of the electropotential readings. Still another girl, who had a history of several unsuccessful inseminations performed later in the cycle and whose husband had a markedly depressed sperm count, became pregnant when the procedure was repeated on the seventh day. Of the 27 patients who have yet to become pregnant, only 5 couples are free of male or female abnormalities.

The results obtained in this infertile group offer circumstantial support only to the contention that the electrovaginal potential recordings detect the

ovulatory date. They are, however, most encouraging, and indicate the need for further substantiation.

The second observation that may be made from the electrovaginal potential recordings is their ability to measure ovarian activity, particularly estrogen output. The clinical confirmation has come in two areas: the study of patients with polycystic ovaries and that of patients with breast cancer.

Polycystic ovaries and ovarian agenesis. A group of twenty-five women with either polycystic ovaries or ovarian agenesis has been studied.

The patient with the symptoms of amenorrhea and the physical findings of a small uterus and underdeveloped breasts, with or without hirsutism, tends to have electrovaginal potential readings in the negative range, which we believe represents estrogenic activity. Tests on this group of patients show no cyclic fluctuation in the electropotential curves. Exploratory laparotomies have confirmed the suspicion of the presence of polycystic ovaries (Stein-Leventhal syndrome). Following wedge resection of the ovaries these patients have resumed normal periods, while many show cyclic variations in their electropotential readings, and a few have become pregnant. The fact that the electropotential in these patients is found in the negative range constantly has been helpful in distinguishing the patient with ovarian aplasia from the girl with the Stein-Leventhal syndrome. Laparotomy confirmed the accuracy of the electropotential readings in 3 patients.

Breast cancer. One of the strongest pieces of circumstantial evidence that the electrovaginal potential records the presence of estrogenic activity is the application of the detecting methods in patients who have breast cancer and intact ovaries.

Forty-five patients with breast cancer and intact ovaries have been studied by Lemon and by our group.⁴ The electrovaginal potential recordings continuing in the negative range were recorded after oophorectomy in about 50 to 60 per cent of the patients, suggesting that the adrenal may take over the function of the ovary for a period of time as yet unknown. Prednisone therapy in doses adequate to suppress adrenal function caused a shift in potential from plus 25 to plus 50 mv in about one half of the cases. The important fact emerging from the study is the observation that chiefly those patients who had readings in the high electropositive or "castrate" range (indicating no estrogenic activity) after either oophorectomy alone or oophorectomy plus prednisone have objective remissions from their neoplastic disease.

Total estrogen assays employing the method of Brown⁵ carried out in conjunction with the electrovaginal potential recordings seem to suggest that the correlation in findings is very close.

A blind study of vaginal smears carried out in connection with the vaginal electropotential readings show a general agreement and close correlation. The results obtained, however, do not provide an exact index of vaginal epithelial response to hormonal stimulation.

The absence of any hormonal activity, which is recorded as positive voltage, on the electrovaginogram is reflected in the smears by the absence of any pre-cornified or cornified cells. Normal cyclic fluctuations seen in the electrovaginal potential readings correlate to moderate and marked cornification in-

dices. However, when sustained low negative voltage is recorded (suggesting excess excretion of estrogen) the cornification index does not show the degree of increase expected.

Conclusion

Although the information gathered from this study is largely clinical, it does provide circumstantial evidence that:

The electrovaginal potential readings do reflect ovarian activity.

Cyclic variations appear throughout the menstrual cycle of the normal woman of premenopausal age.

The cyclic variations disappear in the postmenopausal age group, although electronegative readings may persist, which perhaps is indicative of continued estrogenic activity.

The discontinuity of activity in the electrovaginal recordings at the eighth to tenth day of the cycle appears to coincide with ovulation, as shown by successful conception in over 50 per cent of infertile couples and pregnanediol secretion.

The electrovaginal potential recordings may prove of clinical value in determining the day of ovulation and in evaluating ovarian activity in general and persistence of estrogenic activity in particular.

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Part III. Vaginal Infections

FACTORS RELATING TO MYCOTIC AND TRICHOMONAL INFECTIONS

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A number of infections and inflammatory lesions may involve the vagina. Two unrelated frequent clinical entities, vaginal mycosis and trichomoniasis, are considered here. Each disease will be discussed separately for clarity and because of dissimilarities in pathogenesis. Limitations make review of even the pertinent literature impossible, but other presentations in this monograph supplement this deficiency in references. To the scientifically oriented student it is obvious that certain reports dealing with therapies have been inadequately controlled; for example, a number of microbiological studies have been essentially aerobic in nature. In order to give consideration to factors related to vaginal trichomoniasis and mycosis, statistics on and details of therapy for these conditions have been intentionally omitted.

Since the lay public no longer accepts vaginal discharges and irritation as woman's natural burden, pressure has been exerted for rapid and permanent relief of these conditions. The glamor of the many phenomenal medical advances in other areas in recent years may have contributed to premature reporting, especially in aspects of trichomoniasis. Thus, as the months pass the older of the widely heralded cures tend to disappear. Two questions arise: Is the problem of vaginal mycosis fundamentally one of reinfection? Is failure of trichomoniasis therapy related to the establishment of a normal vaginal flora?

Mycosis

Mycotic infections of the vagina are usually typical in appearance and occur usually during pregnancy. Almost all of the vaginal mycoses are caused by *Candida albicans*. Other fungi may be found infrequently in the vagina. The *Cryptococcus* and *Endomyces* have been recovered from the human vagina only rarely, and other fungi are rarer still. The previous terminologies were medical monilia or *Monilia albicans*. Since a botanical species was also called *monilia*, confusion existed, and this explains the reason for the current name. At one time the words "yeast" or "yeastlike" were common. This was a frank error in terminology. The similarity of the conidia was the initial cause for the term. *C. albicans* produces well-developed mycelia, but the conidia do not yield spores. On the other hand, the true yeasts can form spores and only rudimentary mycelia. The true yeasts are *Saccharomyces*, and are not pathogenic. They are employed extensively for our daily living, home and commercial baking, and breweries and wineries. The error in using the term yeasts caused problems to the commercial producers and users of yeasts because of threats of litigation.

Pathogenicity

The works of Hesseltine *et al.*,¹² Bland *et al.*,⁵ and Hesseltine and Campbell¹³ supplied evidence of the pathogenicity of the *C. albicans* for vaginal mycosis and its relationship to oral thrush of the newborn. Gradually it became known through reports such as those of Benham and Hopkins,³ Halde and Aragon,⁷ and Johnson and Mayne¹⁶ that these organisms may be found commonly in normal humans (skin and nail bed, mouth and intestinal tract, and vagina). The incidence in the vagina is 2 to 3 times higher during pregnancy. Woodruff and Hesseltine²² found ranges from 14 to 41 per cent, with the higher rate related to the underprivileged strata of the population. More than one half of such patients in Woodruff's series were free from symptoms and lacked clinical evidences of mycotic infection. Furthermore, these organisms have been cultured from foods and even from drinking water. This frequency almost places these organisms as ubiquitous. Certainly, the spread to the vagina could be expected inasmuch as the vaginal flora has a relationship in part to that of the intestinal tract. However, contaminated hands or other objects also could be the means of spread to the vagina.

Diagnosis

The diagnosis of vaginal mycosis should present no particular problem. In vaginal mycosis there is ample caseous material or thrushlike patches on the vaginal wall from which to obtain material for a moist-drop examination; if necessary, the material may be dried and then stained by Gram's method. Mycelia are easily recognized, and conidia also can be identified microscopically. Since the carrier state is so common, the cultural method is unreliable and will result in false-positive diagnoses. Cultures from the vagina for fungi are no more diagnostic than are positive cultures for a number of organisms from the nose, throat, or sputum. The laboratory report must be in full compliance with the clinical findings, but it does not displace clinical judgment.

Vulvar mycosis offers other problems diagnostically. The findings of a positive culture of the vulva may be helpful. However, the typical vulvar lesion and symptom complex must be present. The process is confined principally to the mucous membrane of the introitus. It will be a deep tomato red or will have a bluish or gray overcast superimposed on a red background. Vulvar mycosis is limited almost completely to those who have diabetes mellitus in an uncontrolled state. Vaginal mycosis, on the other hand, is found almost always in pregnant patients, and vanishes without treatment a few days after delivery, except in the presence of poorly controlled diabetes. An occasional postmenopausal individual may have sufficient response to estrogens so that a vaginal mycosis develops. The withdrawal of estrogens and subsequent atrophy of the mucosa is paralleled by the loss of the fungous condition.

Symptoms

The report of Adair and Hesseltine¹ indicates that these organisms do not invade the tissues. It is well known that the invariable symptom of vaginal mycosis is a compelling and insatiable itch of the vulva that tends to be worse in the late afternoon and evening. Basically, an "itch symptom" results from

an irritation; consequently, one may suspect that the itch is caused by a by-product of the organisms. Hesseltine¹⁰ quotes Neuberg and Kobel on the point that acetaldehyde, acetic acid, pyruvic acid and other products are formed in the fermentation of carbohydrates. Even diluted solutions of acetaldehyde cause itching of mucous membranes; perhaps this is the agent that gives rise to the distress. The nature of the nerve supply to the vagina may explain the localization of the symptoms to the vulva.

Therapy

The therapy of mycosis has been directed principally toward specific fungicides. Relatively recent attempts to free the intestinal tract of *C. albicans* have been made. However, even if successful this would not prevent these organisms from re-entering the body. The more direct approach has been local therapy and utilization of proper genital hygiene. For a number of years gentian violet was the agent primarily used, generally in concentrations of 1 to 5 per cent in aqueous or glycerin vehicle. One great objection to the use of this dye was that it left an unremovable stain on clothing, floors, and furniture. An even more serious objection was the rare patient who was allergic or sensitive to it. Over the years numerous substances were tested in the laboratories, and the more promising agents were given clinical trials. As so often happens, *in vitro* observations fail to conform in *in vivo* applications. Hesseltine and his colleague^{9, 11, 14} discontinued using a combination of potassium iodate and potassium iodide due to some vaginal irritation from the liberated element iodine.

Fatty acids have fungicidal value, but some cause violent reaction in the vagina. According to reports, both propionic compounds and ricinoleic acid are well tolerated by patients. Alter *et al.*² found propionic acid and salts a good fungicidal agent. Hesseltine and Beckette¹¹ discovered that ricinoleic acid with oxyquinoline produced cures without undesirable side effects. Claims have been made that acids or alkalis may be beneficial. Noonan and Hesseltine¹⁴ showed that the *C. albicans* have a remarkable faculty for growth in pH ranges of 3.5 to 7.5. It was at pH 8.5 and 2.5 that growth failed to occur, although the organisms were not totally killed. In the pH ranges above 4.5 there was lowering of the pH when growth occurred. Thus, the anticipated virtues of acids and alkalis in this condition were disproved.

Predisposing Factors

The disease process is associated with the availability of carbohydrate material, such as the glycogenlike accumulation in the vaginal mucosa in normal pregnancy¹ and glucose in the uncontrolled diabetic.¹³ The vaginal mucosa is atrophic, and glycogen is poor in the normal postmenopausal period, yet under estrogenic stimulation one may produce a mucosa very similar to that of early pregnancy, with moderate amounts of glycogenlike material. This new environment is favorable to the establishment of vaginal mycosis.

If carbohydrate material and common sugars were contributors to mycosis, the deposition of such should enhance mycotic infections. In 14 patients a glucose solution (125 gm. in 1250 cc. water) was poured over the vulva in 250-cc. volumes 5 times daily. Another group of 9 patients had an average

of 6 gm. of glucose deposited in the vagina daily. In these women Hesseltine¹⁰ found that all 13 of the fungifree patients remained symptomfree, while all 9 of those with fungi present developed mycosis or, if the disease was already present in a mild stage, it was made more severe. With the discontinuance of glucose application those who had symptoms reverted to their previous state.

Vaginal Trichomoniasis

Vaginal trichomoniasis as a distinct clinical entity has been recognized for well over thirty years. In the earlier period treatments were few, results were poor, and the concepts of the nature of the disease were mainly theoretical. It is primarily a disease of adulthood and usually confined to the reproductive span. It does occur after menopause, but rarely prior to adolescence. The severe and persevering cases seem more common in those who have good vaginal wall and perineal supports, as well as a normal introitus. However, the disease may occur in the presence of rectocele, cystocele, and prolapse, but less severely as a rule. These observations are in accord with the report of Bland and Rakoff.⁴

Etiological Agents

Controversy has waxed and waned on the causative organism of moniliasis. However, in the typical trichomonadal vaginitis, the vaginal tetratrichomonads and an abnormal bacterial flora for menacmic women are almost invariably present. Whether the flagellates are directly pathogenic or are symbiotic in relationship has not been established. The typical case has a pH in the neighborhood of 6 to 6.5 and the vaginal walls are reddened and often "stippled." The bacterial floral pattern is that of type 2 or 3, according to Schröder, and white cells greatly outnumber the epithelial cells. In the most acute states, epithelial cells will be very scarce or nearly absent.

Some means of spread of the disease are understood. Although the spread may be related to sexual congress, other means of dissemination are thought to be more common. Studies by Trussell and Johnson²⁰ and others suggest that the vaginal trichomonads probably do not reach the vagina by means of the gastrointestinal tract, but the means of spread or possible vector is left in doubt. The disease entity was experimentally produced by Trussell and Plass²¹ through the inoculation of bacteriafree vaginal trichomonadal cultures into volunteer subjects. These inocula included from 200,000 to 2,000,000 organisms. The high percentage of disease response indicated to these investigators that the bacterial flora of the experimental subjects were unrelated to the disease susceptibility. These workers courteously supplied the same culture to Hesseltine *et al.*¹⁵ so that a similar study could be undertaken by the latter team. For this experiment, inocula of 10,000 to 120,000 organisms were used. The investigation was made on 53 volunteer subjects in whom the nature of cell content and bacterial flora was established before and subsequent to the experiment. A total of 7 had the response of the disease or harbored the organisms for a sufficiently long period to be classified as a successful inoculation. Only 2 patients with normal cell content acquired the disease, yet 5 in 23 women with abnormal cellular spread developed the entity. Only 1 patient in

16 with a normal bacterial flora acquired the disease. Meanwhile, 6 individuals among 37 with the abnormal bacterial environment developed the disease. A larger series would have been desirable, but was impracticable. The above reports imply strongly that not only the size of the inoculum, but the nature of the vaginal flora have a relationship to the disease process.

Diagnosis

The diagnosis of the clinical state can be substantiated by the examination of a moist drop in physiological saline solution or plain water. Experienced workers can usually identify these organisms under low-power microscopy, but higher magnification can be used when necessary. Fresh preparation allows observation for activity of the undulating membrane or of the anterior flagella. Papanicolaou and Wolinska¹⁹ reported that their success by the cytological method was quite reliable and probably as good as that of moist preparations. Kean and Day¹⁷ and others have made similar observations, but emphasize that the cultural methods are more laborious and time-consuming than the cytological or moist-smear methods. These data support the fact that the cytological method is effective, but both the cultural and cytological methods require more time than does the examination of the moist preparation. Adair and Hesseltine,¹ in studying the histological picture of the vaginal wall of chronic and severe trichomoniasis, found the mucosa badly broken or destroyed, and localized areas of inflammation were under the mucosal layer. Such sites were found even when the vaginal mucosa seemed normal. Thus, these sub-mucosal lesions might be focal sites from which recurrence could arise. A number of workers have found cervical, urethral, and bladder lesions in patients with chronic or recurrent infections. With the eradication of the inflammation from these areas, some heretofore unyielding cases have become cured.

Over the years many preparations and methods of treatment have been advocated. Enthusiasm has waxed and waned for each of them and, in time, most have been replaced by other methods. Even the recent and current literature too frequently contains papers on the therapy of trichomoniasis without proper controls. In 1937 Hesseltine⁸ stressed the need for controls, pointing out that he obtained results equally as good by the controls as with the specific preparation. The controls were the vehicle alone. The Council on Pharmacy and Chemistry of the American Medical Association⁶ reported: "The most significant aspect of the status of therapy of *Trichomonas vaginalis* vaginitis is that good results ensue from so many substances and variations in the methods of their use and that a small percentage of failures persist in spite of similar treatment. To elucidate these failures is the chief problem." The final paragraph of the report reads: "In view of the fact that *T. vaginalis* cannot be proved pathogenic in every case of vaginitis in which it is found, that unknown local and general factors are operative and that the disease is not likely to be fraught with serious possibilities and may even disappear without treatment, the present aim should be not for new medicaments but for further information, especially concerning failures. In the meanwhile, thoroughness and persistence with the simplest and least messy procedures and a general health program appear to be the therapeutic objects."

The public wants rapid and easy cures and, unless they are obtained, patients may interrupt their course of treatment. Such demands require honest instruction to each patient and the reduction of office visits to a minimum. It is an interesting observation that the pharmaceutical houses that promote new products for trichomoniasis are likely to be the smaller or less well-known ones.

Action of Therapy

The types of treatment could be classified, according to pharmacological and physiological action, into a few categories. Some treatments stimulate the rapid growth of vaginal mucosa. Other methods employ drying or desiccation action of the vaginal walls. A third procedure consists of a moderate escharotic process. Still another attack has been that of antimicrobial action. A fifth approach (postmenopausal group) has been the physiological restoration of the mucosa with increased glycogenlike content. Confusion is increased by the fact that gelatin and glycerin suppositories result in cures. Plain lactose (alpha or beta) has been as efficacious therapeutically as have other agents. One reason for using lactose instead of other sugars is that in the presence of *C. albicans* there is less likelihood of the development of mycosis. These fungi thrive in the presence of a "fermentable" carbohydrate, and lactose is one sugar that they do not "ferment."

Factors of Mycosis

Persistence or recurrence follows improper therapy or premature discontinuance of correct therapy. Because *C. albicans* is so commonly present and thrives at every opportunity (as in pregnancy or uncontrolled diabetic patients), the reappearance of the vaginal mycosis need not be a failure of cure per se; it may be a new infection.

The effort to eradicate all of these organisms from the body seems ridiculous in the light of our present microbiological knowledge. If one can clear the dermal covering, the gastrointestinal tract, and other cavities, what means are available to prevent recontamination from other sources? Frankly, there is almost constant exposure to such probabilities. Consequently, for the present it would seem that therapy of the local process is the only logical course. The next statement is obvious: the therapy must be safe and without undesirable by-products.

It has been demonstrated that those patients with repetition of vaginal mycosis may safely be treated as needed or uninterruptedly, if necessary, up to term. Very few patients will have such persistent mycosis.

Factors in Trichomoniasis

It seems clear that a normal bacterial flora is associated with a normal vagina. It is also known that estrogenic therapy to postmenopausal patients brings about a normal menacmic vaginal mucosa and a corresponding type of flora. Thus, it seems obvious that the establishment of a normal vaginal flora would be an essential part of the treatment. One may then ask how the non-pathogens can be uninfluenced and the pathogens attacked by the use of antibiotics, sulfonamides, and antiseptics.

One problem is the present-day desire to have rapid, miraculous therapeutic response: the public expects it for conditions unrelated to major illness. The use of douches is a mechanical means of removing the evidence without much therapeutic benefit. With the disappearance of symptoms, patients are prone to discontinue therapy and forget the physician. Perhaps each patient needs sincere discussion before the initiation of therapy, with the understanding that it may require weeks to effect a cure. Under pressure, the physician may yield to the temptation of trying other agents too quickly and may overlook situations in the cervix, urethra, or bladder that account for the perpetuation of the illness. The fact that the cure of vaginal trichomoniasis may be rapid in the early or mild case gives no reason to expect the same response in the persistent or chronic one. Lang *et al.*¹⁸ emphasize thoroughness, exactness, and perseverance in the care of each patient.

General Comment

In the studies of organisms, a plea is made that not only aerobes, but also the anaerobes, should be studied. Most studies fail to pay much attention to anaerobic organisms, which are common in the vagina; when one does not recover them, either the techniques are faulty or some error has entered into the study. It is true that the isolation and identification of anaerobes are much more difficult; but such hurdles are the test of competency of the microbiological study.

Certainly, one should avoid using agents in the vagina for either vaginal mycosis or trichomoniasis that may cause bacteria within the patient's body to become resistant, or agents that may result in sensitization or reactions. These would include our more frequently used antibiotics and most of the sulfonamides, as well as a number of chemicals.

Surely, resolutions to the dilemma of poor therapeutic results lie in improvement of the diagnosis, consistency and thoroughness in care, advancements in better therapeutic skills and agents, and the acquisition of further knowledge of the course of vaginal mycosis and trichomoniasis. The exclusions of the diagnostic and clinical errors will be a main step to better therapy. Satisfactory therapy for vaginal mycosis is now available through propionic acid and salts or, perhaps better, by ricinoleic acid and oxyquinoline, but avoidance of reinfection is fundamental. In time, a better management should be evolved for vaginal trichomoniasis. Most probably, it will be concerned with the establishment of a normal vaginal flora.

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METABOLIC STUDIES ON *TRICHOMONAS VAGINALIS*

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At present there is no topical agent capable of effecting a cure of trichomoniasis. Implicit in the term cure is the eradication of the parasite as well as the achievement of clinical relief. Where a systemic agent has demonstrated success to a limited degree, its use has generally been accompanied by undesirable side effects.

The eradication of *Trichomonas vaginalis* as the source of infection remains an insoluble problem, not because the parasite possesses an unusually effective defense mechanism but, paradoxically, because of the protection provided by the host. Elimination of the protozoan from the vagina is a relatively simple task but, once sheltered within the confines of the glands of Skene or Bartholin and other possible sites, the trichomonad becomes almost inaccessible. The usual topical medicants cannot penetrate, and most systemic agents fail to reach the parasite in sufficient concentration to be effective. Since the role of the male is recognized as being of major importance in the transmission of the infection, a systemic approach to the problem of prophylaxis and therapy is essential. The stringent conditions imposed by the location of the parasite within the glands or other sites of the female requires that the effective trichomonadicide possess the attributes of an ideal chemotherapeutic agent; that is, it must be active at very low levels of concentration while not producing any untoward effects in the host.

The search for such a compound has been approached from two directions; a screening program and the study of the nutrition and metabolism of the parasite. The screening program has of necessity assumed the position of primary importance, and justifiably so, since this approach has resulted in the discovery of the principal antibiotic and chemotherapeutic agents of the past two to three decades. Such a program entails a methodical examination of countless chemicals and antibiotic substances both for trichomonadocidal activity and for toxicity to the host. Compounds selected for testing generally fall into one of three categories: first, those that have demonstrated activity against other microbial forms; second, compounds that are structurally related to those having known activity; and third, those selected on a purely arbitrary basis.

The introduction of a program concerned with the investigation of the metabolic processes of the parasite is in no way intended to diminish the importance of screening. On the contrary, it is intended that ultimately the two approaches will complement each other, resulting in a completely integrated project. The initial purpose of the basic metabolic research will be to provide information capable of giving specific direction to the screening program. Eventually, the accumulated data may serve as a guide for the synthesis of new compounds. This tailoring of compounds to meet specific requirements will essentially involve the preparation of analogues, that is, compounds having a chemical composition and structure similar to those that perform some vital function in nature. It is the purpose of these analogues to upset the natural

processes by interfering with the reactions in which the natural compound is essential.

The ability of an analogue to interfere with a vital process cannot, however, serve as the sole criterion for its usefulness. *T. vaginalis* provides a good case in point. Pantothenic acid was reported as being essential for its growth by Kupferberg *et al.* (1948). Further studies by Johnson and Kupferberg (1948) demonstrated that certain of the pantothenate analogues possessed potent trichomonadocidal activity. When tested *in vivo*, however, these compounds were found to be ineffective. The usefulness of the analogue was completely nullified since its purpose, the interruption of pantothenate synthesis, was rendered valueless by the apparent plentiful supply of the preformed vitamin furnished by the host. This aspect of the host-parasite relationship cannot be overlooked where the search for potential inhibitors is concerned.

The search for an effective chemotherapeutic agent depends entirely on the existence of a difference in the metabolisms of the host and the parasite. Discovery of specific differences in the physiologies of the host and parasite will provide information essential to the ultimate solution of the problem.

A further consideration met in the study of chemotherapeutic agents is the extrapolation of *in vitro* results. It must be remembered, and this is particularly true where the oral route is employed, that the agent may be altered by the host and rendered ineffective before it can reach the site of action. Conversely, it has been observed on several occasions that compounds exhibiting little or no significant activity *in vitro* produced a very real protective effect against infection when administered to the host.

The data presented here represent just one phase of our metabolic studies on *T. vaginalis*, namely, the portion that relates to glycolysis and hydrogen transfer.

Almost all of the work discussed here involved the use of cellfree preparations of *T. vaginalis* (axenic strain No. 2). Unlike many other microbial forms, no satisfactory means exists for the preservation of the trichomonad. To maintain the culture in an active, viable state, it was necessary to make transfers every 2 or 3 days. To accumulate sufficient quantities of cells, large-scale culturing of the organism was necessary. Two procedures have been followed, and in both instances the cells were cultured on a STS Medium (Kupferberg *et al.*, 1948) from which the agar and methylene blue had been removed. In the early phases of the investigation the cells were cultured in 4-l. suction flasks, the mouths being fitted with rubber stoppers and the side arms with cotton filters. After inoculation, the air was evacuated from the flask. By the use of this apparatus, the cell populations of 700,000 to 1,200,000 cells/ml. were obtained routinely. Recently, 14-l. fermentation equipment has been employed successfully for the same purpose. In this case, growth takes place in an atmosphere of sterile nitrogen. The population density reached was the same as that obtained in test tube cultures, namely, 2 to 2.5 million cells/ml. A detailed report of this procedure is to be published.

The cells are harvested from the medium by centrifugation and are washed 2 or 3 times with Ringer's solution. The packed cells are maintained at -60° C. overnight and then thawed and ground in a Potter-Elvehjem microhomogenizer. The mixture is then centrifuged to separate the cell debris from

the soluble protoplasmic components. Aliquots, 1 ml. in volume, of the cell-free extract were stored at -60°C . until needed.

A prime consideration in the study of the metabolism of *T. vaginalis* is the fact that the organism is essentially anaerobic; that is, the oxygen concentration must be low if the organism is to survive. Johnson (1942) demonstrated that both the population density and survival were inverse functions of the oxygen concentration. For this reason it is common practice to culture the organism in a medium in which the redox potential has been lowered by means of compounds such as thioglycolate and cysteine. Characteristic of many anaerobes is the absence of a cytochrome system, but the presence of flavine nucleotides that function in the transport of hydrogen. Employing the usual techniques, we have been unable to detect the presence of any cytochrome *c* in *T. vaginalis*. Thus far, there is no report of any of the cytochromes being present in this organism. Through the use of extraction procedures and paper chromatography, both riboflavin and flavin mononucleotide were isolated from the cell. When the cell contents were assayed microbiologically for the presence of riboflavin, a concentration of 75 mg./gm. of cell dry weight was found. This level of riboflavin concentration was not found to differ greatly from that found in bacteria.

T. vaginalis, with all its requirements for a reduced redox potential, has been reported by several investigators (Ninomiya and Suzuoki, 1952; Kupferberg *et al.*, 1953; Read and Rothman, 1955; and Wirtschafter *et al.*, 1956) as taking up oxygen when manometric experiments are conducted with heavy cell suspensions or cell extracts. Such a phenomenon would seem to contradict the basic nature of the organism. Following the lead provided by the report of Mallin and Seeley (1958), who observed this phenomenon in the anaerobic bacterium *Clostridium perfringens*, an investigation was made into the presence of a diphosphopyridine nucleotide (DPNH) oxidase system in *T. vaginalis*. Such a system is capable of oxidizing directly the niacin containing coenzyme DPNH. This system was investigated, employing manometric procedures in which a cellfree extract of *T. vaginalis* served as the supply of the enzyme(s) and reduced DPN as the substrate; the results are presented in FIGURE 1. The oxygen uptake was found to bear a direct relationship to the amount of substrate present. No definitive statement can be made as to whether the oxidation resulted from a single enzyme or group of enzymes, since only the crude preparation was examined. The results are explicit, however, with regard to the uptake of oxygen and its relationship to DPNH oxidation.

A second point of major significance in the metabolism of *T. vaginalis* is the method by which it metabolizes glucose. The organism has been reported by a number of investigators as producing lactic acid. Kupferberg *et al.* (1953) and Read and Rothman (1955) have both made reference to the high concentration of lactic acid produced by *T. vaginalis*. Only lactic acid was detected when the ether extracts of small volumes of the growth medium were chromatographed on paper. When large volumes of medium (of the order of 4 l.) extracted as described by Kupferberg *et al.* (1953) were examined by paper chromatography, three acids were detected: lactic, malic, and a third as yet unidentified (FIGURE 2). The mechanism by which glucose is broken down to form such large concentrations of lactic acid has been reported on by

Wirtschafter and Jahn (1956) and by Wellerson and Kupferberg (to be published). These reports demonstrate that *T. vaginalis* seems to follow the classical Embden-Meyerhof scheme for the degradation of glucose through the various phosphorylated intermediates ending with pyruvate, which is sub-

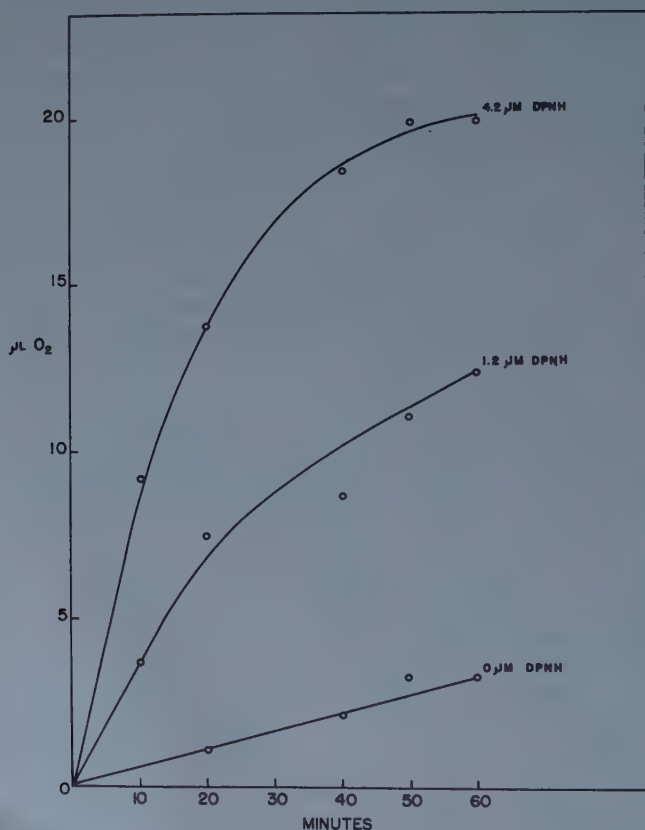


FIGURE 1. The oxidation of DPNH by cellfree extracts of *T. vaginalis*. The test was performed employing standard Warburg respirometer equipment and technique. Contents of reaction flasks (15-ml. capacity): 1 ml. 0.6 M phosphate buffer, pH 6.0; 0.5 ml. cell extract (equivalent of 1.3×10^8 cells), 0.2 ml. 0.001 M MnSO_4 , DPNH (Pabst), 14.2 μ moles/ml., in side arm; 0.2 ml. 30 per cent KOH in center well; total volume brought to 3.0 ml. with distilled water. Conditions: atmosphere, air; temperature, 37° C.

sequently converted to lactic acid. Evidence for the presence of most of the intermediates and enzymes involved has been described.

Recently, we have examined another aspect of metabolism in which lactic acid plays a significant role, namely, that of carbon dioxide fixation (unpublished data). This phenomenon, once thought to be the exclusive property of photosynthetic systems, is now known to exist in protozoa as well as bacteria. Kupferberg *et al.* (1953) reported on the fixation of radioactive carbon dioxide by *T. vaginalis*. Since that time we have been able to isolate and identify the end product of this fixation. The only material isolated that has any

significant radioactivity was lactic acid, and all of the radioactivity was confined to the carboxyl carbon of this compound. The specific mechanism involved in the fixation has not yet been demonstrated. The usual malic acid dehydrogenase and carboxylase systems do not seem likely for the following two reasons: no labeled malic acid was isolated from the system, and the cell extracts tested gave no indication of carboxylase activity. The almost ex-

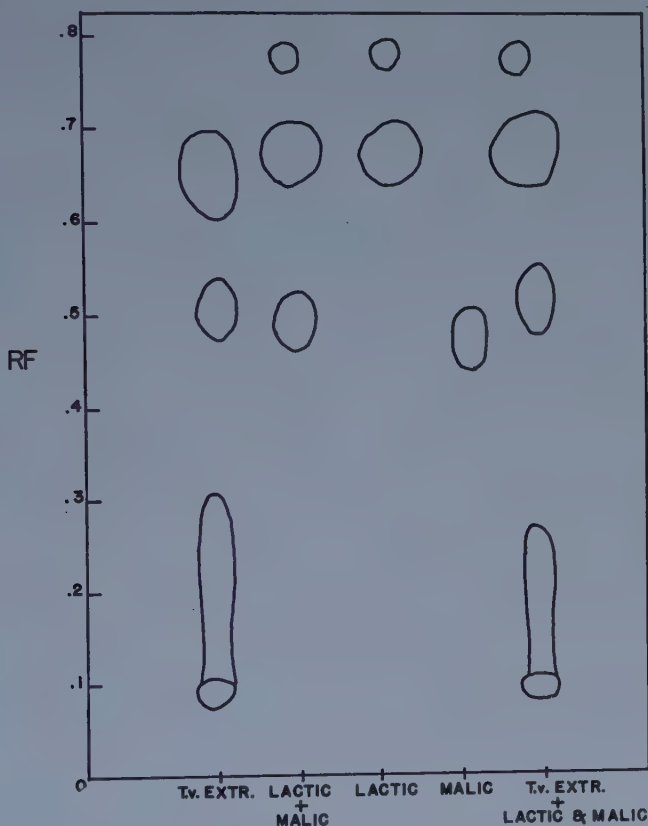


FIGURE 2. A paper chromatogram of the acids produced by *T. vaginalis* during growth. The chromatogram was run ascending at 24° C. for 16 hours. Solvent: butanol, 95; formic acid, 5; saturated with water. Paper, Whatman No. 1. The paper was dried, steamed gently, and sprayed with a bromphenol blue indicator (45 mg./100 ml. ethanol), pH 7.3.

clusive labeling of lactic acid in the carboxyl carbon in spite of a prolonged exposure (48 hours) suggests a mechanism involving an entry into the Embden-Meyerhof scheme. The ribulose-1,5-diphosphate-carbon dioxide fixation mediated by a carboxylation enzyme is offered as a possibility.

This report integrates some of the accumulated data concerned with one phase of the metabolism of *T. vaginalis*. It represents an infinitesimally small portion of the over-all physiology of the organism, yet through studies of this type it is hoped that sufficient data will result to provide a means for effectively eliminating the problem of trichomoniasis.

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NONSPORE-FORMING ANAEROBIC BACTERIA OF THE VAGINA

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During the preadolescent and postclimacteric ages the vagina is the normal habitat for bacteria commonly referred to as the nonspore-forming anaerobic group. During menstrual life, the vaginal incidence of these same bacteria depends on the types of patients cultured, on the cultural methods used, and on the incidence of associated vaginal infections. It is also known that nonsporulating anaerobic bacteria represent a large part of the bacterial flora of the lower intestinal tract, and are frequently, if not always, present in the upper respiratory tract.

The term nonspore-forming separates these bacteria from the spore-forming anaerobic *Clostridia*, which are more familiar to most clinicians. The anaerobic cocci are morphologically similar to aerobic cocci and are grouped by Bergey¹ with the aerobic cocci in the genera *Streptococcus*, *Micrococcus*, *Diplococcus*, and *Neisseria*. An additional Gram-negative coccus, which may be of importance, is placed in the genus *Veillonella*. Bergey¹ regards all anaerobic, nonspore-forming, Gram-negative bacilli as species of *Bacteroides*. Although not recognized by Bergey,¹ the Gram-positive, nonspore-forming, anaerobic bacilli from the vagina and elsewhere are usually regarded as species of *Corynebacterium* (diphtheroids).

Most American bacteriologists follow Bergey's¹ system of taxonomy and nomenclature, whereas, many European investigators follow a system advocated by Prevot.² The two groupings are similar, with two major exceptions: first, Prevot recognizes a number of species of Gram-positive, nonspore-forming, anaerobic bacilli. Some of these form gas in culture and produce a fetid odor. For the lack of a better name we have regarded these organisms as anaerobic *Corynebacteria*. Prevot² places them in the family *Bacteriaceae* Cohn emend., under the genera *Eubacterium* Janke 1930 emend., *Catenabacterium* Prevot 1938, *Ramibacterium* Prevot 1938, and *Cillobacterium* Prevot 1938. The second exception is that Prevot,² on the basis of morphology, divided the *Bacteroides* of Bergey¹ into two orders. Species not considered pleomorphic are placed in the order *Bacteriales*, whereas pleomorphic strains are believed to be closely related to the *Actinomycetes*, and are placed in the order *Actinomycetales*. On the basis of morphology, motility, capsule formation and other properties, a number of genera and species has been devised by Prevot. A more complete discussion of *Bacteroides* was published by Carter *et al.*³ in 1953.

From the standpoint of practical identification, neither Bergey's nor Prevot's groupings of the supposedly adequately studied species are of value to the bacteriologist working in obstetrics and gynecology. This deficiency is due to the fact that much of the work within this field has been done by clinicians or bacteriologists working with a single strain or with small numbers of strains. Emphasis in obstetrics and gynecology, as well as in other fields, has been on naming new species rather than in developing uniform methods applicable to the entire group. Many of the descriptions of the reported species are

inadequate to the degree that too often an organism is rediscovered by a subsequent investigator and is given a new name. No two workers use the same technique for identification, and there is no accurate comparative basis for the identification of newly isolated strains. With our present knowledge, the specific species identification of a single organism is a research problem and not within the scope of the routine bacteriological laboratory. Over the years an additional factor that has contributed to the problem is that only rarely are adequate methods of culture used. Furthermore, with the best cultural methods known, some nonspore-forming anaerobic organisms grow well only in symbiosis with other anaerobes, and others fail to survive lyophilization or pure-culture transplant for a sufficient length of time to permit detailed studies. As a consequence, only those organisms that can be grown and that can be transplanted have been studied. In our opinion, the organisms in this category represent only a fraction of those that may be of importance in obstetrics and gynecology.

Since the discovery of antibiotic and chemotherapeutic agents the problems associated with the treatment of the venereal diseases and with a majority of the infections due to aerobic bacteria have been markedly reduced. During this same period, little progress has been made in the treatment of the incapacitating group of anaerobic infections that involve the female reproductive system. It is our belief that nonspore-forming anaerobic bacteria cause major problems in obstetrics and gynecology that require further fundamental research. If directed toward a clearer understanding of their cultural requirements, methods of lyophilization, and species identification, basic studies would be invaluable if carried out in a uniform fashion.

Methods of Routine Culture

Unusual precautions are necessary for the successful cultivation of nonsporulating anaerobic bacteria. For the best results, material must be cultured as soon after collection as possible; either excessive drying or prolonged exposure to oxygen may be lethal. This is particularly true when only small amounts of material are available for culture or when collections are made on cotton swabs. Larger amounts of material can be collected in tubes, placed in the refrigerator, and cultured satisfactorily for as long as 24 hours. We make it a practice either to have the bacteriologist collect all cultures or to make the person collecting the material responsible for transporting it to the bacteriological laboratory immediately after collection.

Most anaerobic infections are due to a mixture of anaerobes. Experience has shown that the primary cultures are most successful if cultures are grown in symbiosis on a medium that favors anaerobic growth over that of the aerobic forms. Some media are selective, and do not meet these requirements. Another important factor appears to be the amount of inoculum. With small amounts of inocula or when materials containing a small number of organisms are cultured, there may be either no growth or a prolonged lag period. For these and other reasons we have found that an anaerobic meat medium is best for primary culture and shows less selectivity; also, the growth shows more correlation with direct smear findings. The formula for the medium we use is a slight modification of that originally described by Lepper and

Martin.⁴ The meat used is neutralized beef heart, and the medium has a final pH of approximately 7.4. Cultures in this medium have the advantage of being sealed by a petrolatum seal that, when broken, permits the detection of gas formation and of odor. Gas formation is of value in the differentiation of some of the anaerobic bacteria from morphologically similar aerobes. For isolation and study, transplants from the primary mixed cultures are made to blood plates, and the hydrogen-replacement method is used.

Routine Practical Terminology

With our present knowledge, detailed studies on individual organisms are both impracticable and impossible. Our bacteriological laboratory uses the terms mixed anaerobes, anaerobic streptococci, and anaerobic cocci. In addition, the presence of pleomorphic *Bacteroides* is noted, and the *Clostridia* are identified when present. The same material is used for both anaerobic and aerobic cultures. Comparison of the two types of cultures determines the necessity for further studies to prove the presence or absence of nonsporulat-

TABLE 1

Possible genera		
Anaerobic nonspore-forming group (mixed anaerobes)	<i>Micrococcus</i> <i>Diplococcus</i> <i>Streptococcus</i> <i>Neisseria</i> <i>Veillonella</i> <i>Bacteroides</i> <i>Corynebacterium</i>	According to Bergey ¹

ing anaerobic bacteria. The term anaerobic implies that an organism is an obligate anaerobe; that is, it will never grow in the presence of oxygen. It is likely that in our mixed cultures some microaerophilic organisms are included. The oxygen and cultural requirements of many of these bacteria need further clarification.

It has been shown by others, and it has been our own experience, that some anaerobic cocci considered as Gram-positive are actually Gram-variable. Despite the age of the culture or the technique used, some pure cultures show degrees of staining variability. TABLE 1 shows the generic possibilities when Bergey's¹ grouping is followed and the term mixed anaerobes is used. This form is used when cultures contain a mixture of anaerobic cocci and anaerobic nonsporulating bacilli, either Gram-positive, Gram-negative, or both. With the exception of the pleomorphic *Bacteroides*, no effort is made to separate the various morphologic types of cocci and bacilli. The term anaerobic *Streptococcus* is used when anaerobic cocci are found that form chains and are streptococci morphologically. The species possibilities, according to Bergey,¹ are shown in TABLE 2. The term anaerobic cocci is used when the anaerobic broth cultures show only a mixture of anaerobic cocci, either Gram-positive, Gram-negative, or both. These bacteria may be of varying morphologic types. The generic possibilities are shown in TABLE 3.

*The Importance of the Nonspore-Forming Anaerobic Bacteria in
Obstetric and Gynecologic Infections*

From an examination of the literature it is difficult to determine the overall incidence of the nonspore-forming bacteria in infections in obstetric and gynecologic patients. This difficulty is apparently due to the fact that adequate cultures have rarely been used. Based, however, on the results obtained in our laboratory, which has been a part of our department of obstetrics and gynecology for many years, it is our belief that these bacteria are the primary cause (and are associated as secondary causes) in the majority of infections in obstetric and gynecologic patients. Furthermore, we believe that further basic knowledge concerning these organisms is vital to a clearer understanding

TABLE 2

	Possible species	
Anaerobic streptococci (anaerobic cocci in chains)	<i>S. anaerobius</i> <i>S. foetidus</i> <i>S. putridus</i> <i>S. lanceolatus</i> <i>S. micros</i> <i>S. parvulus</i> <i>S. evolutus</i> <i>S. intermedius</i>	} According to Bergey ¹

TABLE 3

	Possible genera	
Anaerobic cocci (mixed anaerobic cocci)	<i>Micrococcus</i> <i>Diplococcus</i> <i>Streptococcus</i> <i>Neisseria</i> <i>Veillonella</i>	} According to Bergey ¹

of some of the common infections of the female in which the specific etiological agents are obscure or ill defined.

The vagina. All of the studies on the normal vaginal flora, including our own investigations,⁵ are incomplete because in none of these studies are the anaerobic bacteria separated or identified. It is likely that these organisms constitute the major part of the normal flora during the preadolescent and postclimacteric ages. During the age of ovarian function they can be isolated from the majority of patients who have other than a pure *Lactobacillus* (Döderlein's bacillus) flora.

Vaginitis. Scant attention has been paid to the anaerobes as a possible cause of nonspecific vaginitis. When this diagnosis is made in the premenarchal age group by the exclusion of specific agents as a foreign body or gonococcus, we have found the predominating flora to be anaerobic.⁶ In severe forms of nonspecific vaginitis occurring during menstrual life and simulating clinical trichomoniasis, such as frothy discharge, odor, and petechiae, we find the same predominating anaerobic flora. The nonsporulating anaerobic bacteria in mixed culture (and in many instances in pure culture) are charac-

terized by the formation of gas and by a foul odor. Both characteristics are commonly associated with the trichomoniasis and nonspecific vaginitis syndromes. It is probable in both trichomoniasis and nonspecific vaginitis that the bacteria are equally responsible for the type of discharge produced. Whether the anaerobes occurring in these conditions are identical with those isolated from apparently normal vaginas remains to be determined by further studies. We have been unable to confirm a single organism as the cause of nonspecific vaginitis.

Bartholinitis and skenitis. With the decrease in the number of patients with gonococcal infections, the nonsporulating anaerobic bacteria are the most common organisms associated with abscesses of Bartholin's and Skene's glands. One of the first descriptions of *Bacteroides funduliformis* was offered in 1898 by Halle,⁷ and this organism was obtained from Bartholin's abscesses, from retained placentas, and from healthy vaginas. Although *Bacteroides* usually occurs in association with anaerobic cocci, we have recovered, in pure culture from Bartholin's abscesses, organisms answering the descriptions of *B. funduliformis*.

Pyometra. In 1951 Carter *et al.*⁸ reported the cultural findings in 133 patients with pyometra. Predisposing associated diseases were squamous carcinoma of the cervix, adenocarcinoma of the uterus, senile stenosis of the cervix, tuberculosis of the endometrium, postoperative stenosis of the cervix, and collections of pus in the postoperative cervical stumps. The importance of the anaerobic bacteria in pyometra was shown clearly. Of the 133 patients with pyometra from whom cultures were obtained, only 21 of the cultures were negative. Fifty-one of the patients had mixed aerobic and anaerobic infections. Anaerobic organisms alone were cultured from 49 patients. In the entire series only 12 patients had positive cultures for only aerobic organisms. There seems to be little question that anaerobic bacteria are responsible for much of the secondary infection and odor associated with advanced carcinoma, and frequently are factors in the complications associated with irradiation and operative therapy.

Pelvic inflammatory disease. Pelvic inflammatory disease other than that due to the tubercle bacillus or the gonococcus is generally a symbiotic anaerobic infection due to anaerobic Gram-positive and Gram-negative cocci, various species of *Bacteroides*, aerobic or anaerobic *Corynebacteria* (diphtheroids) or organisms of the coli-aerogenes group. Infections are most frequently due entirely to anaerobes.

Infections may follow gonorrhea, infected abortions, puerperal endometritis, carcinoma, dilatation and curettage, tubal insufflation, pelvic operations, cervical cauterization or conization, or any other invasion of the uterus or pelvis. It is assumed, but not proved, that the vagina is the usual source of the infection, and that the cervix is the common portal of entry. Whereas such an assumption is probably correct, it has not been shown definitely that the anaerobic organisms from pelvic infections are identical with those from normal or infected vaginas.

Postpartum and postabortal endometritis. Since the discovery of penicillin we have routinely cultured the lochia or uterus on all morbid postpartum and postabortal patients. In this group, the number of febrile patients with evidence of endometritis as the probable reason for the febrile reaction runs to

well over one half of the patients cultured. The incidence of such specific organisms as *Streptococcus pyogenes* (Group A), coagulase-positive *Staphylococci*, *Clostridium welchii*, or *Neisseria gonorrhoeae* has been relatively low. On the other hand, the incidence of the nonspore-forming anaerobic bacteria is so high that their isolation is of significance only when more specific pathogenic organisms are excluded by culture and the clinical diagnosis is obvious.

Anaerobic endometritis appears to be dependent not only on the presence of potentially pathogenic anaerobic bacteria in the vagina, but also upon the condition of the patient, the type of delivery, the drainage of the uterus, retained secundines and, possibly, other factors that cannot be measured with accuracy. Fortunately, in term deliveries, when the uterus is completely evacuated, most infections are mild. In incomplete abortions, however, the endometritis is usually more severe and may lead to bacteremia, to peritonitis, to abscess formation, or to chronic pelvic inflammatory disease. Whether only certain anaerobic bacteria are pathogenic or potentially pathogenic is not known.

No controlled studies have proved the value of antibiotics or sulfonamides in these infections when other factors are not considered. It is quite possible that the use of antibiotics in patients with proved anaerobic endometritis constitutes indiscriminate therapy.

Discussion

The so-called "nonspore-forming anaerobic bacteria" of the human vagina form a large group of Gram-positive, Gram-negative, and Gram-variable cocci and bacilli concerning which we have little information that would permit specific species identification. The multiplicity of names in the literature is sufficient evidence of the confusion that exists regarding their isolation and identification. Since these bacteria are not cultured routinely in many bacteriological laboratories, their actual incidence and importance in infections occurring in obstetric and gynecologic patients has not been stressed. No systematic studies have been made in obstetrics or gynecology on a workable classification and identification of the nonspore-forming anaerobic bacteria found in normal or diseased vaginas or from minor or major infections. Until adequate methods of culture and identification of these organisms are developed, their actual role in a variety of common infections that occur in females will be difficult to determine.

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THE VAGINAL FUNGI

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In the ages of ovarian function the vagina is the normal habitat for saprophytic and potentially pathogenic yeasts and yeastlike fungi.^{1, 2} Because the same fungi are not found in the vagina of preadolescent and postclimacteric patients, it is probable that the incidence during menstrual life is related to the increased glycogen content of the vaginal mucosa. A higher incidence occurs during pregnancy, and attempts have been made to explain this phenomenon on the basis of higher hormone levels that tend to produce more mucosal glycogen and to increase the average vaginal acidity. Some investigators³ believe that the so-called "renal glycosuria of pregnancy" may be a contributing factor. *In vitro*, the growth of yeasts and yeastlike fungi is stimulated by the addition of glucose. This factor appears to be the logical explanation for the occurrence of vaginal fungi in diabetics of any age group.

The Vaginal Fungi

Despite the fact that a number of fungi has been isolated from the vagina, only the yeasts and yeastlike fungi appear to be a part of the normal flora or capable of producing vulvovaginitis.

In 1929, Castellani⁴ listed the following genera isolated from the vagina and studied in his laboratory; *Monilia* (*Candida*), *Cryptococcus* (*Torula*), *Saccharomyces*, *Oidium* (*Geotrichum*), *Aspergillus*, *Sterigmatocystis*, *Penicillium*, *Cladosporum*, *Alternaria*, and *Hemispora*. The last six of these are molds and are common contaminants. Vaginal infections with molds have been described, but apparently are extremely rare. Of the yeasts and yeastlike fungi, the *Cryptococci* and *Candida* appear to be of importance, but there are no well-documented reports of vaginal infections by *Saccharomyces* or *Geotrichum*. The importance of the *Saccharomyces* is assumed because they occur in a small percentage of normal vaginas and, when grown on Sabouraud's agar, may simulate species of *Candida*. The gastrointestinal tract is the normal habitat for fungi belonging to the genus *Geotrichum*. However, these fungi have not been isolated in our laboratory from the vagina in several thousand vaginal cultures.

The three common genera, *Candida*, *Cryptococcus*, and *Saccharomyces*, all grow readily on Sabouraud's agar at room temperature. They produce colonies that have a yeasty odor and are not unlike colonies of *Staphylococci*. As a rule, when first isolated, the three genera cannot be differentiated on Sabouraud's agar. Exceptions are the red or pink *Cryptococci*, and the occasional saccharomycete that forms ascospores on this medium. In old cultures on Sabouraud's agar, some *Candida* will form mycelia. Such formation usually occurs in the upper, dried-out portion of the agar slant. Reproduction by all three genera is principally by budding, and as the cells enlarge they become detached from the parent cell and continue the budding process. Microscopic examination reveals only round or oval cells or blastospores.

On appropriate media the *Saccharomyces*, or so-called "true yeasts," produce

ascospores that are the sexual and perfect stage of these fungi, which places them in the class Basidiomycetes. Ascospores develop inside large, thick-walled cells called asci, and vary from 2 to 8 in number. Conjugation occurs within the ascus, with primary nuclear fusion and reduction of chromosomes.

Candida and the *Cryptococci* are commonly referred to as yeastlike because blastospores are produced in the same fashion and are of the same type as those produced by the Saccharomycetes. However, they are placed in the class Fungi imperfecti, because the perfect (sexual) stage has not been observed with certainty in most of them.

The Genus Candida

The genus *Candida* reproduces by the formation of blastospores. On corn meal agar or other appropriate media a true mycelium or pseudomycelium is formed that grows down into the medium and is vegetative in type. In direct preparations from the vagina both the mycelial elements and yeast cells are usually visible.

The type, and most important, species of the genus is *C. albicans* (Robin) Berkout 1923. Since Robin's⁵ original description in 1853, much of the confusion regarding the genus has been caused by the innumerable names given this fungus by different investigators. The extent and amount of confusion are best shown by the number of apparently synonymous terms found in the literature by different reviewers. According to Skinner (1947),⁶ Cifferi *et al.* (1938) found 45 Latin binomial synonyms for *albicans*; Diddens and Lodder (1942)⁷ found 87, and Conant (1939)⁸ stated that there were 172 in the literature.

The generic terminology has been changed frequently, but in the medical literature the most common generic term used has been *Monilia*. This term, first used by Hill in 1751 for an unrelated group of fungi, has been shown by a number of investigators to be incorrect when applied to the so-called "medical monilias." The confusion regarding correct generic terminology for this genus was so great that, at the Third International Microbiological Congress in 1939, delegates voted and agreed to use the generic term *Candida* Berkout 1925. Excellent cooperation has followed the action of the congress, but some mycologists^{6, 8} still believe that *Syringospora* Quinquaud 1868, has priority over the name *Candida*.

There is a number of authentic species of *Candida*, but the number of species isolated from human sources is relatively small. Diddens and Lodder,⁷ studying strains from all sources, recognized 25 species and 8 varieties, with the majority of the species from sources other than human. On the other hand, Martin *et al.*⁹ studied 461 strains isolated from humans, and only 6 valid species were recognized. Four additional strains were studied that differed from each other, but the authors hesitated, without further study, to give them species rank and to add more names to the literature. The 6 common species isolated were identified as shown in TABLE 1.

Since the reports of Martin *et al.* (1937)⁹ and of Jones and Martin (1938),¹⁰ the identification of *Candida* isolated from the vagina by the Bacteriologic Laboratory, Department of Obstetrics and Gynecology, Duke University Medical Center, has been a routine procedure. Of the hundreds of vaginal

strains studied, 5 of the 6 common species have been found to occur in the vagina, the only exception being *C. pseudotropicalis*.

The Genus Cryptococcus

The *Cryptococci* are simple yeastlike organisms. Reproduction is by budding; the cells enlarge, become detached from the parent cell, and continue the reproductive process. On corn meal agar or carrot plugs neither a true mycelium nor ascospores are produced. On corn meal agar some strains may produce elongated blastospores that simulate the mycelia produced by *Candida*.

The only proved human pathogenic species of the genus is *Cryptococcus neoformans* (*Torula histolytica*), which produces infections of the brain, meninges, lungs, skin, or other parts of the body. True cryptococcosis has a widespread geographical distribution, and has been reported from various parts of the world. Evidence points to a single species that is characteristically pathogenic for small laboratory animals. The fungus produces a wide capsule under certain conditions of cultivation and in tissue.

TABLE 1
RECOGNIZED SPECIES OF *CANDIDA*

<i>C. albicans</i>	233 strains (50.5 per cent)
<i>C. stellatoidea</i>	110 strains (23.5 per cent)
<i>C. parakrusei</i>	47 strains (10.2 per cent)
<i>C. krusei</i>	36 strains (7.8 per cent)
<i>C. tropicalis</i>	28 strains (6.7 per cent)
<i>C. pseudotropicalis</i>	3 strains (0.7 per cent)
<i>Candida</i> sp. (unclassified)	4 strains (0.3 per cent)

Supposedly nonpathogenic *Cryptococci* commonly occur in the vagina and on the vulva and have been isolated from diseased and normal skin and from other various sites on the human body. The pathogenicity of any of the *Cryptococci* other than *C. neoformans* has to be proved by further study.

Species differentiation by the use of fermentations, as has been applied to *Candida*, has not been done to any great extent with *Cryptococci* isolated from humans. In most studies, in the absence of specific infections, *Cryptococci* have been regarded as nonpathogenic saprophytes. In the studies that have been made on human strains a number of different species can be identified on the basis of carbohydrate fermentations and other tests.

The red-pigmented *Cryptococci* commonly occur in fungus cultures as air contaminants. Unless a fair number of colonies is present or unless colonies can be found in the cultured material by direct examination, they should always be regarded as contaminants.

The Genus Saccharomyces

The ascosporogenous yeasts occur occasionally in vulvovaginal cultures, but are less common than are the yeastlike fungi. These fungi are important because in freshly isolated Sabouraud's agar cultures they multiply only by budding and therefore resemble *Candida* and *Cryptococcus*. On appropriate

media, however, and occasionally on Sabouraud's agar, they produce ascospores. Such spores develop inside large, thick-walled cells called asci and may be from 2 to 8 in number. They are called sexual spores because they develop as a result of nuclear fusion, with a reduction of chromosomes. Attempts to identify the different species of *Saccharomyces* isolated from humans are rarely made, since these organisms are regarded as saprophytes and have not been shown to produce infection.

Differentiation of Genera

Generic differentiation of the vaginal yeasts and yeastlike fungi is based on morphology under methods of cultivation designed to force production of the differential characteristics of mycelial production and ascospore formation. FIGURE 1 shows the morphology of the three common genera as they occur under special conditions of cultivation and in direct preparations from the vagina. In culture, *Candida* occurs in two distinct phases that depend on the

	Direct Examination of Vaginal Discharge	Morphology in Culture
Cryptococcus		
Saccharomyces		
Candida (Monilia)		

FIGURE 1. The 3 genera of vaginal yeasts and yeastlike fungi, showing morphologic differences in preparations direct from the vagina and from special media.

environmental factors of available oxygen and available food. On highly nutritious media, under aerobic conditions, only blastospores are produced. However, reduced oxygen tension and the lack of an abundant food supply favor the formation of mycelia and, in certain species, of chlamydospores. One of the common media used that favors mycelial and chlamydospore formation is corn meal agar. Cultures done on this medium should be grown at room temperature and inoculated by deep stab or by cutting into the agar with a straight inoculating needle. When plate cultures are made, it is wise to overlay the inoculated streak with molten agar. Carrot plugs are usually satisfactory in forcing the production of ascospores in the *Saccharomycetes*. Regardless of the type of medium on which they are grown, the *Cryptococci* produce only blastospores.

Description and Methods of Identification of the Vaginal Candida

The medical bacteriologist or mycologist concerned with *Candida* isolated from human sources is more interested in the characters that aid identification than in variations that occur on different media or under different conditions of cultivation. It is known that dissociative or degenerative changes occur

spontaneously in old cultures, and that such changes can be induced by the use of chemicals or immune serum.^{11, 12} Before working with old cultures one should become familiar with freshly isolated strains maintained by frequent transplants. When it is necessary to attempt to identify old cultures it is a wise procedure to attempt to reverse changes that might have occurred. For this purpose we have found beef extract with sheep's blood agar a valuable medium, since changes in colony characteristics are easily detected in it. Cultures on blood agar should be planted from Sabouraud's glucose broth. The smoothest colonies are picked and are planted again in Sabouraud's broth, and the procedure is repeated. Reversion is not likely to occur unless some evidence of it is noted in the first 2 or 3 transplants. Incubation of blood agar plate cultures should be at 37° C. The use of blood agar in studying *Candida* has been considered unusual by some mycologists, but it is more familiar to bacteriologists who have been trained in the observation of bacterial colonies.

It has long been known that morphology alone could not be used to separate different species of *Candida*. Benham, in 1931,¹⁴ was one of the first to advocate a combination of characters, and she was able to define most of the medically important *Candida*. Following Benham,¹⁴ Jones and Martin¹⁰ and Martin *et al.*⁹ described methods of identification that could be successfully applied to the medically important *Candida*. These methods have been in use in our own laboratory with only slight modifications for a number of years and, when applied to the vaginal *Candida*, have proved satisfactory.¹⁵

No single procedure can be relied upon for identification of all the vaginal *Candida*. Nevertheless, with experience, rapid identification with considerable accuracy is possible by using only morphology, colony formation on blood agar, and type of growth in Sabouraud's broth. This statement applies to freshly isolated vaginal strains, and is not made for strains from other sources. It has been found that most of the variations that occur in freshly isolated vaginal strains can be accounted for by differences in composition and pH of the various substrata, methods of inoculation, amount of available oxygen, and the temperature of cultivation. The methods of preparing the necessary media are included in the APPENDIX.

Outline Identification Procedures

In outline form the following procedures are used: (1) isolation of fungus on Sabouraud's glucose agar; (2) type of surface growth in Sabouraud's glucose broth after incubation for 48 hours at 37° C.; (3) types of colonies produced on beef extract sheep's blood agar plates at pH 7.4, inoculated from Sabouraud's broth, and incubated at 37° C. for 10 days or less; (4) single colony picked from blood agar plate and transferred to Sabouraud's glucose agar; (5) some of the same growth is transplanted to carrot plugs for possible formation of asci, and the remainder streaked on beef extract agar pH 7.4 and transferred for 2 to 3 generations; (6) growth from beef extract agar streaked on corn meal agar slide cultures for details of morphologic growth; (7) growth from beef extract agar inoculated into beef extract broth tubes containing bromthymol blue indicator and 2 per cent glucose, sucrose, lactose, and maltose. The carbohydrate broth is sealed with sterile petroleum jelly and is incubated at 37° C.

for 10 days or less. The inoculum from the beef extract agar should be a saline suspension; (8) intravenous inoculation of rabbits with a 1 per cent saline suspension of fungi.

Growth on Sabouraud's Agar

All of the vaginal *Candida* grow well on Sabouraud's agar, and this medium is satisfactory for their isolation. Growth is usually apparent in from 24 to 62 hours, depending on the species, and growth is good at room temperature or at 37° C. Species differentiation is not possible on this medium, since only blastospores are formed in the early stages of growth. All species save *C. krusei* form white to grayish, raised creamy colonies. A yeasty odor is found in all species. Colonies of *C. krusei* are drier and flatter on Sabouraud's agar than are colonies of other vaginal species. Although an occasional short strand of mycelium may be seen on Sabouraud's agar, as a rule in the blastospore stage, only the pigmented *Cryptococci* can be definitely separated from *Candida* and *Saccharomyces*. In general, the *Cryptococci* and *Saccharomyces* from the vagina produce smaller blastospores than do the *Candida*, and the cells of *C. krusei* are more elongate. These differences, however, are not of sufficient specificity to permit separation.

Growth in Sabouraud's Broth

Sabouraud's broth serves 3 important purposes in the study of *Candida*, permitting (1) purification of cultures where bacteria occur as contaminants; (2) observations on the type of surface growth produced; and (3) the inoculation of blood agar plates for colony observation.

Broth cultures should be incubated at 37° C. for 48 hours before observations are made for surface growth. It is obvious that the tubes should not be shaken or disturbed during this period of incubation. If the tubes are incubated for a longer period, the procedure loses diagnostic value. In cultures of *C. krusei* a dry-appearing surface film is formed that extends up the sides of the tubes to a distance of 5 to 6 mm. above the surface of the medium. Due to vigorous fermentation, surface growth occurs to a lesser degree with *C. tropicalis*. In the latter fungus, however, the surface growth consists mostly of numerous small bubbles of gas, and is not so characteristic as is the growth of *C. krusei*. No surface growth is produced by the other 3 species of vaginal *Candida*. Characteristic growth may or may not be obtained if bacterial contaminants are present, and the cultures should be repeated until a pure culture is obtained.

Colony Formation on Blood Agar

The formation of reasonably typical colonies on beef extract sheep's blood agar is extremely valuable in identifying the vaginal *Candida*, provided six precautions are taken in the techniques: (1) the agar should be fresh and reasonably soft, and the plates should be freshly poured; (2) the inoculum should be from Sabouraud's broth or other glucose media, and the fungus should be primarily in the blastospore stage; (3) colonies should be well isolated; (4) the fungus should be freshly isolated and should not show evidence of dissociation; (5) all plates should be incubated at 37° C.; (6) if *C. tropicalis*

is suspected, the plates should be incubated for 10 days, as this is the period required for the development of a mycelial fringe; in other species characteristic colonies will be formed in 3 to 4 days. The following descriptions present only those features that have proved to be the most constant and valuable in species identification.

C. albicans. Well-isolated colonies grow in 3 to 4 days, are approximately 1.5 mm. in diameter, are more or less circular in outline, and have a smooth border. The surface is slightly convex, and the colony is a dull grayish white. The rate of growth in 24 hours is greater than that seen in *C. stellatoidea* and will be obvious if the 2 species are compared on blood agar after 24 hours' incubation. In poured blood agar plates a slight greenish blue tinge is produced that is much less than that seen in *C. stellatoidea*. When the medium has become dehydrated to any extent, the colonies may be less round and a few projections may occur. These, however, are never so great as are similar projections produced by *C. stellatoidea*.

C. stellatoidea. This species has a slower rate of growth on blood agar than *C. albicans*. The colony is composed of an elevated central zone from which tapering arms radiate in an irregular manner. The projections are made up of budding cells on the surface and mycelia beneath the surface. In poured blood agar plates a distinct green color is evident around the colonies.¹⁶ Surface colonies are star-shaped, as the species name indicates.

C. parakrusei. Well-isolated colonies are round, have a smooth border, and are quite convex. Their color is pearly white, and they average 0.6 to 0.8 mm. in diameter, which is smaller than in the growth of *C. albicans*.

C. krusei. Colonies of this species are characterized by variations in size and shape that occur in colonies equally well isolated. The size varies from 0.2 to 1.0 mm., and the borders may be round or irregular. The surface may be smooth, ridged, nodular, flat or heaped.

C. tropicalis. Colonies of this fungus are identical in color and shape to *C. albicans* in that they are round and whitish gray in color; however, they are somewhat larger, averaging 2 mm. in diameter. After 6 to 10 days of incubation, the colony is surrounded by a mycelial fringe, and the mycelia form a zone approximately 1 mm. in width around the colony.

Carbohydrate Fermentations

Only 4 carbohydrates, glucose, sucrose, maltose, and lactose are necessary in identifying the *Candida* isolated from human sources. However, in order to obtain consistent results, a rigid technique must be followed. It has been shown that, when a completely aerobic technique with the use of Dunham tubes is employed, consistent results are not obtained. Furthermore, the use of an inoculum from Sabouraud's agar slants appears to affect the results.

The seven following points in technique are emphasized:

(1) The fungus must be of a pure strain and free of bacterial contamination. This can best be accomplished by culturing on blood agar and picking colonies rather than relying entirely on growth on Sabouraud's agar.

(2) The inoculum should be a saline suspension of the second or third transplant on a sugar-free medium. We employ beef extract agar slants, but other carbohydrate-free agar can be used.

(3) The carbohydrate broth is beef extract broth (see APPENDIX) accurately titrated to 7.2 before the bromthymol blue indicator is added. The sterile carbohydrate solution is added in a concentration of 2 per cent after sterilization.

(4) Breakdown of the carbohydrates should be avoided by sterilizing in solution by filtration.

(5) If the carbohydrate broth is kept for a long period before use, the pH should be checked, since minor change will affect the results.

(6) The fermentation tubes should be sealed with a sterile mixture of petroleum jelly and paraffin.

(7) Incubation should be at 37° C. for 10 days.

The seal prevents fading of the indicator after acid is produced. Reversal of color may occur, however, if the gas pressure in the tube displaces the seal and allows air to come in contact with the culture. In most species the fermentation is complete in several days. Fermentations of the 5 vaginal species of *Candida* are shown in TABLE 2.

TABLE 2
FERMENTATIONS OF THE 5 VAGINAL SPECIES OF *CANDIDA*

	Dextrose	Sucrose	Maltose	Lactose
<i>C. albicans</i>	AG	A	AG	—
<i>C. stellatoidea</i>	AG	—	AG	—
<i>C. tropicalis</i>	AG	AG	AG	—
<i>C. krusei</i>	AG	—	—	—
<i>C. parakrusei</i>	AG	—	—	—

It should be noted that the fermentations of *C. albicans* and *C. stellatoidea* are very similar, as are the morphologies of these two species. With this method, *C. krusei* and *C. parakrusei* ferment the same sugar. Other differences, however, when correlated with the fermentations, allow separation of these species. The lactose fermenting *C. mortifera* has not been isolated from the vagina in our laboratory, but was one of the species recognized by Martin *et al.*⁹ For that reason we include lactose in our fermentation tests.

Mycelial Production and Morphology

The demonstration of mycelia is necessary to separate *Candida* from the *Cryptococci* and *Saccharomyces*. Mycelial production in *Candida* is influenced by the age of the growth, by the moisture content of the medium, by the amount of available food, by the temperature of incubation, and by the amount of available oxygen. In general, on highly nutritious media only blastospores are produced under aerobic conditions. When mycelia are produced, morphology is also affected by the same factors. The development of chlamydospores by *C. albicans* and *C. stellatoidea* is important in identification, and sharply separates these two species from all the other *Candida*. For mycelial production and observations on morphology, corn meal agar is the satisfactory medium. Cultures can be done on slides in a moist sterile chamber

or in Petri dishes that can be examined directly with the low-power microscope. The fungi can be stained with lactophenol cotton blue on slides exactly as they grow, which is an ideal method when large numbers of strains are studied. Cultures for studying morphology should be incubated at room temperature, and mycelia are more readily formed when the inoculum is from sugarfree media. Regardless of whether plates or slides are used, partial anaerobic conditions are created by flooding the inoculated area with molten agar which aids mycelial production. The following descriptions are based on only the most constant and characteristic features presented by each species.

C. albicans produces a well-developed, branching, treelike mycelium with numerous chlamydospores; as a matter of fact, in some laboratories the production of chlamydospores is regarded as specific for *C. albicans*. This is more likely to be true for strains from sources other than the vagina, since *C. stellatoidea* cannot be separated from *C. albicans* morphologically. Under certain conditions clusters of blastospores develop along the mycelial threads, and this is more liable to occur in strains that do not readily form mycelia. The production of mycelia, both as to number and ease of production, is a variable factor in all *Candida* and must be kept in mind when these fungi are studied.

C. stellatoidea cannot be differentiated from *C. albicans* morphologically. In general, fewer chlamydospores are formed, and more ball-like clusters of blastospores are found.

C. parakrusei produces mycelia with difficulty and, when mycelia are produced, they have more of the characteristics of pseudomycelia than those produced by other species.

C. tropicalis forms mycelia more readily and more profusely than any of the other vaginal species. The hyphal elements project a considerable distance from the line of streak. Numerous blastospores are found scattered throughout the mycelial branches. Chlamydospores are not formed.

C. krusei, in most cultures, forms fairly well-developed mycelia that appear as naked threads branching only at wide intervals. The blastospores are longer and narrower than in the other species, and when they occur in masses they resemble a bundle of crossed sticks. Chlamydospores are not formed.

Pathogenicity Tests

When injected intravenously, freshly isolated strains of *C. albicans* kill rabbits in 4 to 5 days or sooner. This test differentiates this species from all the other vaginal species save *C. tropicalis*, which has been reported to kill if it is injected in enormous doses. However, when 1 cc. of a 1 per cent solution of packed fungus cells is used, only *C. albicans* is pathogenic for rabbits.

Summary of Differential Characteristics

The following summary describes the most important differences between the closely related vaginal species. Since we have not isolated lactose-fermenting *Candida* from the vagina, only three carbohydrates—dextrose, sucrose, and maltose—are considered.

C. parakrusei. This organism forms a creamy colony on Sabouraud's agar, but no surface growth in Sabouraud's broth. The colonies are pearly white,

round colonies on blood agar; mycelia form with difficulty on corn meal agar; acid and gas form in glucose; maltose and sucrose are not fermented; it is not pathogenic for rabbits.

C. krusei. This organism forms flat, dry colonies on Sabouraud's agar; it causes extensive dry surface growth in Sabouraud's broth; there is irregularity in size and shape of colonies on blood agar; the mycelia show few branches; blastospores may be arranged like crossed sticks on corn meal agar; acid and gas are formed in glucose; sucrose and maltose are not fermented; it is not pathogenic for rabbits.

Although these two species have identical fermentations, differentiation is relatively easy. The dry type of growth on Sabouraud's agar and the dry surface growth on Sabouraud's broth are particularly characteristic for *C. krusei*. Morphologically, mycelia are more readily formed, and the blastospores are typically more elongate in *C. krusei*. In addition to these differences, on blood agar *C. parakrusei* produces rounder, more uniform, and pearly white colonies. Neither fungus is pathogenic for small laboratory animals.

C. albicans. This mold forms creamy colonies on Sabouraud's agar; there is no surface growth in Sabouraud's broth; medium-sized dull gray colonies on blood agar are formed that are usually round, but may show irregularity that is never as great as in *C. stellatoidea*; chlamydospores are formed in corn meal agar; acid and gas are formed in glucose, and maltose and acid in sucrose; it is pathogenic for rabbits.

C. stellatoidea. This mold forms creamy colonies on Sabouraud's agar; there is no surface growth in Sabouraud's broth; stellate colonies are formed on blood agar, and their rate of growth is slower than that of *C. albicans*; chlamydospores are less readily formed on corn meal agar than in *C. albicans*; there is acid and gas formation in glucose and maltose and no fermentation of sucrose. Green pigment is produced around colonies in poured-blood agar plates, and is much more intense than the pigment produced by *C. albicans*. *C. stellatoidea* is not pathogenic for rabbits.

C. albicans and *C. stellatoidea* are closely related both morphologically and culturally; however, differentiation is easily performed by the comparison of rate of growth and types of colonies on blood agar. Pigment production occurs in poured-blood agar plates. Fermentation of sucrose occurs. There is pathogenicity of *C. albicans* for rabbits.

C. tropicalis. This species forms creamy colonies on Sabouraud's agar; the surface growth on Sabouraud's broth is characterized by a narrow film and by gas bubbles due to vigorous fermentation; the early colonies on blood agar are round and resemble *C. albicans* in color, but are slightly larger; a late mycelial fringe is produced around the colonies; mycelia form readily and are profuse in corn meal agar; chlamydospores are not produced; it ferments all three sugars with the formation of acid and gas; it is not pathogenic for rabbits save in massive doses.

Cryptococcus sp. This organism forms either creamy or pigmented colonies on Sabouraud's agar; it does not produce mycelia or ascospores on any type of culture medium. On corn meal agar, blastospores may elongate and form chains that can simulate the mycelia produced by *Candida*. By far the

greater majority of all vaginal strains studied by us will not grow, or do so but poorly, on blood agar.

Saccharomyces sp. This organism forms creamy growth on Sabouraud's agar; it does not produce mycelia; it occasionally produces ascospores on Sabouraud's agar, but more commonly on carrot plugs or other special media; most vaginal strains grow poorly or not at all on blood agar; it is not pathogenic for animals or humans.

The failure of most vaginal strains of *Cryptococcus* and *Saccharomyces* to grow on blood agar gives value to this medium for the study of the vaginal fungi since failure to grow automatically eliminates any of the vaginal *Candida* that are known to us.

Incidences

The incidence of fungi in the vaginas of normal pregnant patients has been found by all investigators to be higher than that in normal nonpregnant patients. However, the number that develop symptomatic vaginal infections is low in relationship to the number of carriers. If all pregnant patients with fungi in the vagina were symptomatic, the problems of prenatal care would be materially increased.

Ch'in and Lim¹⁷ in 1936 cultured the vagina of 100 asymptomatic pregnant patients and found positive cultures on 39. These investigators recognized 13 different species of *Candida* and used Castellani's method of classification, which has since been shown to be incorrect. In 1938 Woodruff and Hesseltine¹⁸ found in the last trimester of pregnancy an over-all incidence in the vaginas of 20 per cent. In 1939 Waters and Cartwright¹⁹ found an incidence of 10.8 per cent. Carter *et al.*¹ found a vaginal incidence during pregnancy of 33 per cent and a vulvovaginal incidence of 43 per cent.

Many investigators have found *C. albicans* in normal stools. There seems to be evidence that there is an increased incidence in patients with certain forms of diarrhea and in those who develop diarrhea while receiving antibiotic therapy. Ashford^{20, 21} found *Monilia psilosis* (which is synonymous with *C. albicans*) in 55 per cent of patients with tropical sprue and in 6 per cent of healthy individuals. Other workers have also found *C. albicans* with other species and genera of fungi in normal stools. Benham and Hopkins²² found *C. albicans* in normal stools of 18 of 100 normal patients, Schnoor²³ found 17 per cent in 314 patients; Negroni and Fischer²⁴ found 14 per cent in 207 patients; Lawler *et al.*²⁵ found 15 per cent in 100 patients.

The oral cavity also seems to be a normal habitat, and a great number of workers have noted the presence of *C. albicans* in normal mouths.²⁶⁻²⁸ Some reports stress a higher incidence in mouths containing carious teeth^{29, 30} and in a common condition known as perleche.³¹

Species of *Candida* other than *albicans* and species of *Cryptococcus* (*Torula*) have frequently been isolated from normal skin. Benham and Hopkins,²² Croft and Black,³² and Mackinnon³³ have all failed to find *C. albicans* on normal skin. A single exception is Marwin,³⁴ who reported an incidence of 1.5 per cent. It is doubtful that the skin is a common source of infection unless it is diseased.

Oral Thrush in the Newborn

Hausmann³⁵ (1869) reported that he had successfully produced mycotic vulvovaginitis by inoculating the vagina of a patient with the thrush fungus *Oidium albicans*. Apparently the inoculation was direct from mouth to vagina, but it was the common belief during this period that oral thrush in the newborn was due to infection from the vagina of the mother.

The first investigators to produce oral thrush in newborn infants with pure cultures of vaginal fungi were Hesselstine *et al.* (1934).³⁶ These investigators used a culture identified as *Monilia pinoyi* (Castellani) or *Candida albicans*, and they inoculated the mouths of 7 newborn infants and produced oral thrush in all of them. Four unidentified strains produced thrush in 2 infants when inoculated into the mouths of 12. These investigators were unable to produce thrush with *Monilia krusei* (*C. krusei*) or *Saccharomyces cerevisiae*.

Bland *et al.* (1937)³⁷ produced mycotic vulvovaginitis in 7 of 12 patients by inoculating the vagina with fungi isolated from patients with oral thrush.

From the above evidence it seems certain that the vagina is the most likely and the most common source of infection. However, under present conditions of hospital practice, oral thrush of the newborn is relatively rare. Woodruff and Hesselstine (1938)³⁸ found an incidence of but 1 per cent in 14,640 consecutive deliveries at the Chicago Lying-in Hospital, Chicago, Ill. In a relatively benign infection, such a low incidence would hardly justify an attempt to eliminate the sources of infection in asymptomatic mothers.

Diabetic or Mycotic Vulvovaginitis

For almost a century it has been recognized that fungi commonly occur in the vagina or on the vulva of diabetic patients with vulvovaginitis. Friedreich (1864),³⁹ writing on diabetes, stated that the itching experienced by diabetic patients was due to fungus organisms. He reported the finding of fungi in 12 diabetic patients and believed these organisms never occurred in healthy patients.

In 1876 Hausmann⁴⁰ and Winckel⁴¹ engaged in a considerable controversy concerning the incidence and the importance of fungi in vulvovaginitis in diabetic patients. Winckel claimed a low incidence of fungi and believed they were secondary in producing the symptoms, which were actually produced by the diabetes itself, whereas Hausmann believed that the fungi were constantly present and of etiological significance.

Wiltshire (1881),⁴² in some clinical remarks on the diagnosis and treatment of pruritus vulvae, stated: "Sugary urine obviously supplies a most favorable pabulum for the development of the lowly organized fungi." He suggested that pruritus vulvae associated with vulvovaginitis should lead to the suspicion of diabetes and stated that *Oidium albicans*, the thrush fungus, had been found.

In 1933 Hesselstine⁴³ reported the isolation of fungi from 18 of 21 diabetic patients with vulvitis. He believed that the so-called "diabetic vulvitis" was always a mycosis, and he was unable to produce vulvitis or pruritus in fungus-free patients by the use of a solution of glucose as a vulvar wash; however, he was able to produce severe pruritus in a patient with fungi in the vagina and with a mild pruritus. The fungi isolated were identified as *Saccharomyces* 1,

Cryptococcus 1, *Endomyces* 6, *Monilia* (*Candida*) 1, undetermined 3, lost 2, not listed 4. He later classed the fungi considered to be *Endomyces* as *Monilia* (*Candida*).

In 1937, Hesseltine⁴⁴ again emphasized the belief that the term diabetic vulvitis was incorrect and reported the isolation of *Cryptococci* or *Monilia* (*Candida*) from 54 of 57 diabetic patients with vulvitis. However, from diabetic patients without vulvitis he found an incidence of but 12.5 per cent.

Summary

The yeasts and the yeastlike fungi are the only fungi that commonly can be cultured from the vagina and from the vulva. The incidence of these fungi appears to be related to ovarian hormonal function, to the administration of ovarian hormone, to pregnancy, and to diabetes.

The three genera that can be cultured from the vulva and the vagina are *Saccharomyces*, *Cryptococci*, and *Candida* (*Monilia*).

The true yeasts of the genus *Saccharomyces* are the ones least frequently found in cultures. These fungi appear to be saprophytic and have not been proved to cause disease of the human vulva and vagina.

The simple yeastlike fungi of the genus *Cryptococcus* are frequently cultured from the vulva and vagina. These yeasts do not cause the same changes in the vulval and vaginal tissues as do the yeasts of the genus *Candida* (*Monilia*). The role that the yeasts of the genus *Cryptococcus* may play in the production of pruritus must be determined by further investigation.

The most important genus is *Candida* (*Monilia*). At least five or more species have been cultured from the vulva and vagina. Of these species, *C. albicans* is the most important in the production of symptomatic mycotic vulvovaginitis. A closely related species, *C. stellatoidea*, is found most commonly in asymptomatic pregnant patients.

An effort has been made briefly to review some of our knowledge of the yeasts and the yeastlike fungi.

APPENDIX

Sabouraud's glucose agar. Bactoglucose, 40 gm.; Fairchild's peptone, 10 gm.; agar, 20 gm.; distilled water, 1000 cc. Melt in autoclave, filter through cotton, tube, and sterilize in autoclave at 15-lb. pressure for 15 min. No pH adjustment is necessary.

Sabouraud's glucose acid broth. Same formula as for Sabouraud's glucose agar, but no agar is added. No filtration or pH adjustment is necessary.

Beef extract agar. Difco beef extract, 3 gm.; NaCl, 5 gm.; Difco peptone, 10 gm.; agar, 20 gm.; distilled water, 1000 cc. Melt in autoclave, titrate to pH 7.6, filter through cotton, tube, and autoclave at 15-lb. pressure for 15 min. Final pH, 7.4.

Beef extract blood agar. Same basic formula as for beef extract agar with the addition of approximately 10 per cent sterile citrated sheep's blood.

Carbohydrate broth. Difco beef extract, 3 gm.; NaCl, 5 gm.; Difco peptone, 10 gm. Make to 900 cc. in distilled water. Heat to boiling and titrate exactly to pH 7.2. Add 100 cc. of indicator solution (see below), filter, and tube in

10-cc. quantities. Autoclave at 15-lb. pressure for exactly 15 min. Add 1 cc. of a 20-per cent solution of the carbohydrate sterilized by filtration through a Seitz filter.

Indicator solution. Bromthymol blue, 0.04 gm.; distilled water, 100 cc. Add a small amount of 1 normal NaOH to make the solution alkaline. When the indicator is in solution, neutralize with 1 normal HCl until the exact neutral point is reached, and add 1 drop of either acid or alkali to cause a complete change in color.

Corn meal agar. Add 62.5 gm. corn meal to 1500 cc. water. Heat to 60° C. for 1 hour. Filter through paper and make volume up to 1500 cc. Add 19 gm. of agar. Arnoldize for 1¼ hours. Filter through cotton, tube, and sterilize. No adjustment of pH necessary.

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HEMOPHILUS VAGINALIS VAGINITIS

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That a widespread, nontrichomonal, malodorous leukorrhea exists that has been attributed in the past to "nonspecific" bacterial vaginitis is undeniable. Clinical descriptions of "nonspecific" vaginitis appearing in the literature are rare and grossly inadequate, but the clinical pattern is far more uniform than that observed in vaginal trichomoniasis or moniliasis. The etiology of non-specific vaginitis has been postulated in the past, although seemingly contrary to the usual rules of medical logic, as being of mixed bacterial character, with the various streptococci, staphylococci, micrococci, diphtheroids, and coliform bacilli being most often incriminated. In a preliminary report in 1954¹ and a more detailed report in 1955² we presented evidence that over 90 per cent of so-called nonspecific vaginal infections belong to a specific disease entity caused by a single bacterial species, namely, *Hemophilus vaginalis*. Our additional years of experience have added only confirmation to our previously published opinions, and these opinions have also been largely confirmed by Lutz *et al.*,³ Ray and Maughan,⁴ Amies and Jones,⁵ Brewer *et al.*,⁶ and Stewart.⁷ Deming⁸ described a closely similar, if not identical, entity but identified his causative bacterium as *H. influenzae*. Heltai and Taleghany⁹ reported isolating *H. vaginalis* 30 times from 127 clinic patients but, according to their material and methods, were unwilling to assign *H. vaginalis* a more significant role than numerous other organisms. Consequently, they questioned the existence of a specific entity and preferred to adhere to the concept of a mixed bacterial etiology. Hunter and Long¹⁰ apparently failed to identify the organism in a group of 87 patients.

Materials and Methods

Selection of patients. The present study encompasses 371 pregnant and non-pregnant white patients from a private office practice with a diagnosis of *H. vaginalis* vaginitis. A few years ago we would have classified the entire group as having nonspecific vaginitis. Diagnoses were made only after careful correlation of clinical, microscopic, and bacteriological findings. The statistical results were formulated from 940 independent clinical observations (in the 371 patients), made on days when vaginal material was tested for hydrogen ion concentration and studied both microscopically and bacteriologically. Patients were excluded from the study if they yielded *Trichomonas vaginalis* or species of *Candida*; also excluded were patients with purulent cervicitis and vaginal bleeding from any source. Most observations were made after abstinence from douching and intercourse for three or more days. Since estrogen-deficient patients generally do not demonstrate classic findings of the common vaginitides, only women in the childbearing age who had not recently aborted or delivered were included. For a variety of reasons, immediate postsurgical patients were not included. The necessity in some instances, and the desirabil-

ity in all, of adhering to these rules in selecting patients for a study of this nature requires no detailed explanation.

Laboratory methods. During the years minor changes in methods have been instituted. Material for culture was taken on a small, dry, sterile, cotton-tipped applicator from the vaginal vault ahead of the spread blades of an unlubricated bivalve speculum. After material was obtained for culture, the speculum was then inserted the full distance of the vagina, and as much material as possible was collected on the posterior blade. The material on the speculum was observed for volume, color, consistency, odor, and frothiness. Using Hydrion paper, pH was determined from the pooled material on the speculum. Slides for staining were made in the routine manner. Wet-mount preparations were made by mixing a small amount of the vaginal material with a drop of isotonic saline on a glass slide, and a coverslip was applied. In recent years we have used wet-mount preparations as a routine screening procedure and consider it the most reliable single laboratory test for the differential diagnosis of vaginitis.

Cultures. Material obtained from the vaginal vault was emulsified in 0.5 to 1.0 ml. thioglycolate broth, Brewer-modified without indicator (BBL). The emulsified material was transferred within 3 to 4 hours to solid media with a sterile cotton swab. The swab was rolled over the diameter of the plate, and the deposited material distributed by streaking at right angles with a wire loop. Cultures for trichomonads were made only when indicated.

The most efficient medium found to date for the isolation and maintenance of *H. vaginalis* is Casman's blood agar base (Difco) adjusted to pH 7.5 prior to autoclaving and containing 5 per cent fresh defibrinated rabbit blood. Isolation of *H. vaginalis* is more consistent when the cultures are incubated under increased CO₂ tension (candle jar) for 24 hours at 37° C.

Clinical Findings

In order to determine accurately the true clinical picture of this disease it was necessary to eliminate patients yielding other obvious pathogens such as *Trichomonas* and *Candida* (see *Materials and Methods*, above, describing the prerequisites for selection of patients).

Volume of discharge. This varies from scanty to profuse, but usually it is considerably less than in the average case of trichomoniasis. The majority of cases will show a slight discharge, some a moderate, and a few a profuse discharge. Many patients, while being unaware of or denying a discharge, will admit soiling of the underwear and the necessity of frequent douching.

Consistency of discharge. The discharge is of approximately the same fluidity as that of trichomoniasis. It has some resemblance to a thin flour paste, being turbid but uniformly homogeneous and with a tendency to adhere to the vaginal wall in a thin film instead of pooling in the posterior fornix. Brewer and his associates described the discharge as "smooth," while others have described it as "creamy." Although it definitely does not have the usual curdy consistency of normal vaginal secretions, it was described as slightly curdy in 5.5 per cent of our observations. The vaginal secretions rapidly assume a normal curdy consistency with eradication of *H. vaginalis*.

Color of discharge. In this study the discharge was recorded as gray in 94.9 per cent of the cases, white in 2 per cent, and yellowish or greenish in 3.1 per cent. Brewer and his associates described the discharge as white or gray in 89.2 per cent, yellow in 6.4 per cent, green in 2.2 per cent, and clear in 2.2 per cent. In trichomoniasis, the discharge has either a green or a yellow cast in approximately 50 per cent. Normal secretions are usually gray, white, or slate-colored.

Odor of the discharge. The odor of the discharge, while being characteristic, is usually less offensive than that of trichomoniasis. On 771 occasions (82 per cent) it was recorded as disagreeable and 169 times (18 per cent) as offensive. Brewer and his associates described the discharge as "uniformly disagreeable." Practically all patients, when questioned directly, will admit awareness of a disagreeable odor. The characteristic malodor disappears with the eradication of *H. vaginalis* by appropriate treatment.

Frothiness of the discharge. In the earlier series of 141 cases of *H. vaginalis* vaginitis,² 27 per cent exhibited some degree of frothiness but, in the present study, frothiness was recorded in 17.7 per cent of the observations. Brewer and his associates found frothiness in 21.3 per cent. The presence or absence of frothiness is not constant for individual patients. While frothiness in trichomoniasis is more common and frequently more pronounced, it no longer can be considered a pathognomonic sign of trichomoniasis.

The pH of vaginal discharge. Eighty-two per cent (771) fell within the range of pH 5.0 to 5.5, while 71 per cent (669) fell within the narrow range of pH 5.0 and an estimated 5.2. Only 18 per cent (169) showed a reading of less than pH 5.0, and of these only 3.4 per cent (32) fell below an estimated pH 4.7. Brewer and his associates reported an average of pH 5.2 for their series. For comparison, a series of 100 consecutive control patients (without cervicitis) considered to have normal vaginal secretions, with lactobacilli and/or diptheroids as predominant organisms on smear and culture, was tested. All readings were within the range of pH 3.8 to 4.5, and 85 per cent fell within the range of pH 4.0 to 4.2. We have observed that patients in the childbearing age with vaginal secretions of pH 5.0 or greater and with no trichomonads will have *H. vaginalis* vaginitis in more than 90 per cent of the cases. A determination of pH is easily, quickly, and inexpensively performed, and yet is of real diagnostic significance. Eradication of *H. vaginalis* results in a return to normal of the vaginal pH.

Subjective symptoms. Subjective vulvovaginal irritations associated with *H. vaginalis* vaginitis are in no way comparable to those associated with acute trichomoniasis or moniliasis. In the earlier series² we reported that 31 per cent of patients had mild itching or burning of the vulva, while in the 940 observations of this study, subjective symptoms were recorded 122 times (13 per cent). Brewer and his associates reported that 44.7 per cent of their patients with *H. vaginalis* vaginitis had symptoms other than discharge, while Ray and Maughan reported subjective symptoms in 38 per cent.⁴ A significant number of patients with clinically and bacteriologically normal vaginas will answer affirmatively when directly questioned about vulvovaginal irritations.

Objective signs. In the earlier series² we reported that 22 per cent of patients

had a minimal vulvitis as indicated by redness or edema, and that 11 per cent had evidence of vaginal pathology as indicated by petechiae or redness. In the present study, gross tissue changes were recorded 68 times (7.2 per cent). Ray and Maughan reported that only 5 of 68 patients had redness or edema,⁴ while Brewer and his associates reported that 34 of 47 patients had redness or punctate areas of the vagina.⁶

Laboratory Findings

Wet mounts. The wet mount in *H. vaginalis* vaginitis presents a characteristic picture (FIGURE 1). Pus cells are few in number, being quite rare in some

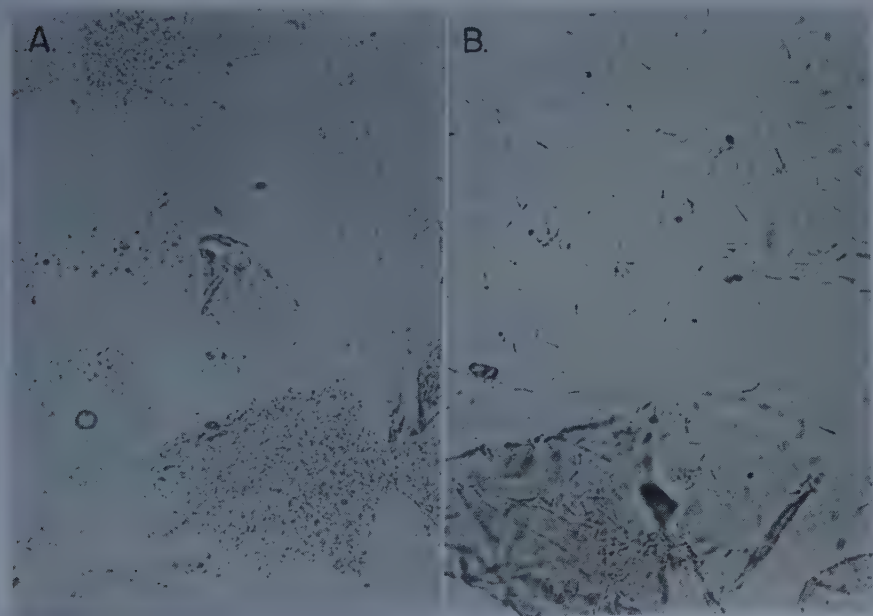


FIGURE 1. A, wet mount, showing "clue cell" of *H. vaginalis* vaginitis (lowermost structure). $\times 302$. B, wet mount showing normal secretions with lactobacilli. $\times 302$.

instances unless there is a concomitant infection or incidental cervicitis. Lactobacilli are conspicuously absent. The appearance of the epithelial cells in the wet mount provides the most valuable clue to the presence of *H. vaginalis*. The cells assume a stippled or granulated appearance due to the adherence and uniform spacing of *H. vaginalis* upon the surface of many of the cells. Not all cells are involved, and some of the affected cells show only partial involvement. A few organisms, mainly diphtheroids and cocci, at times will adhere to epithelial cells and mimic the "clue cell" of *H. vaginalis*, but we have little difficulty in differentiation. Frequently, clumps of *H. vaginalis* are observed attached to the edges of epithelial cells or floating free in the surrounding fluid. A motile organism that appears to be a vibrio has been found associated frequently with *H. vaginalis*.

Stained smear. The most striking feature of the Gram-stained smear is the presence of *H. vaginalis*, which is seen in tremendous numbers as small Gram-negative bacilli with rounded ends, 0.3 to 0.6 μ wide and 1.0 to 2.0 μ long (FIGURE 2). The majority of stained smears will reveal one or more other types of bacteria, although the small Gram-negative bacilli usually outnumber all other organisms manyfold. Lactobacilli, as in the wet mount, are conspicuously absent.

Cultures. Microbiological cultures are not essential for differential diagnosis in most cases of vaginitis. A careful correlation of clinical findings with wet-mount and stained-smear observations will provide a correct diagnosis in most

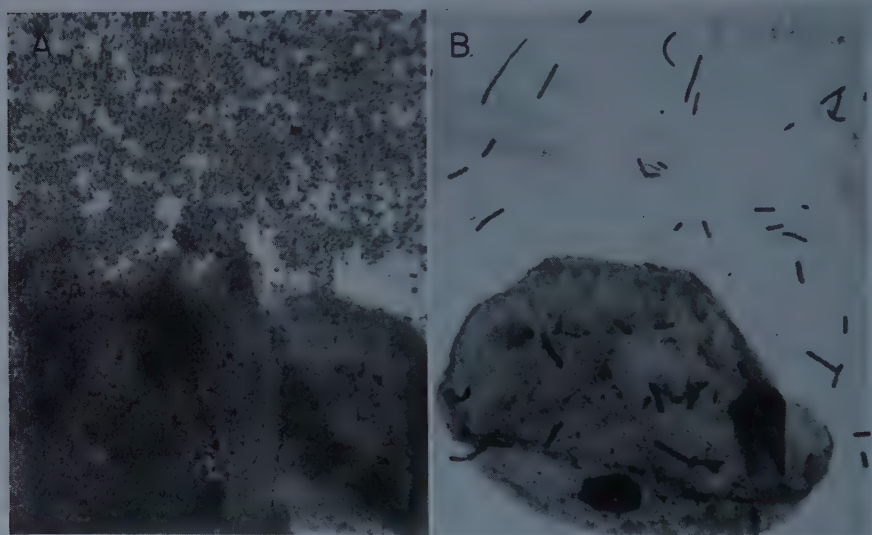


FIGURE 2. A, Gram-stained smear from discharge of *H. vaginalis* vaginitis, showing small Gram-negative bacilli; B, Gram-stained smear from secretions from normal vagina, showing large Gram-positive lactobacilli.

instances. On the modified Casman's blood agar *H. vaginalis* appears as a minute colony 0.05 to 0.5 mm. in diameter. Under low-power stereoscopic magnification ($\times 36$) by reflected light, the colonies appear as smooth, glistening, convex, transparent, circular, entire-edged, and of the dewdrop type, tending to coalesce when in close proximity to one another. Colonies of *H. vaginalis* can be readily differentiated from the more granular, erose, and dull-surfaced vaginal diphtheroids and streptococci that are frequently comparable in colony size. Microscopically, *H. vaginalis* is a Gram-negative bacillus with rounded ends, 0.3 to 0.6 μ wide and 1.0 to 2.0 μ long, occasionally showing a tendency toward pleomorphism, end-to-end pairing, and parallel arrangement.

Isolation of *H. vaginalis* from mixed cultures frequently can be obtained by single-colony transfer to fresh Casman's rabbit blood agar plates. In our laboratory, reference stock cultures have been maintained for over 200 transfers by transferring every 48 hours (24 hours at 37° C. and 24 hours at 3° to

8° C.). *H. vaginalis* is oxidase-negative and in CTA medium (cystine-trypticase agar, BBL) produces acid from glucose, maltose, dextrin, and starch, exhibits no action on lactose, sucrose, mannitol, trehalose, glycerol, raffinose, salicin, or dulcitol, and yields slow and weak acid in xylose.

Pure cultures of *H. vaginalis* may be preserved for periods exceeding 6 months by freeze-drying in sterile rabbit serum. For reconstitution of dried cultures, a broth is prepared according to the Casman formula¹¹ with the omission of the agar, and the addition of 5 per cent fresh defibrinated rabbit blood. Dried cultures suspended in this broth and incubated at 37° C. produce a uniformly turbid growth in 24 to 48 hours. Such cultures, when transferred to Casman's blood agar plates, yield typical morphology.

The 940 vaginal cultures in this report were obtained from 371 patients considered to have *H. vaginalis* vaginitis as determined by clinical signs, microscopic findings, and cultures. Of the 940 independent cultures, 898 (95.5 per cent) were positive for *H. vaginalis*.

Of the 940 cultures, 181 (19 per cent) yielded pure cultures of *H. vaginalis* on the initial plate. In an earlier publication² we reported that 60 per cent of patients yielded pure cultures on one or more occasions, but the majority of these patients had been cultured from 3 to 12 times.

Incidence

While the present study does not permit a determination of incidence, we recently reported the screening of 2251 white private patients with the finding of one or more types of vaginitis in 23.2 per cent.¹² *H. vaginalis* was isolated from 12.2 per cent of all patients studied and from 52.3 per cent of all patients with vaginitis. Trichomonads were present in 8.1 per cent of all patients studied. A simultaneous survey of 993 clinic patients showed that 69.3 per cent had one or more types of vaginitis, and of the total patients studied, *H. vaginalis* was present in 29.1 per cent, or in 42.0 per cent of all patients with vaginitis.

Ray and Maughan in an analysis of 447 clinic patients found *H. vaginalis* vaginitis present in 15.2 per cent, or in 44.2 per cent of all patients with vaginitis. In addition, they reported that of 72 patients considered to have bacterial vaginitis, 68 cases were attributable to *H. vaginalis*. In a study of 211 patients with leukorrhea, Brewer and his associates recovered *H. vaginalis* in 89 (42.2 per cent). In a preliminary analysis Stewart⁷ has found *H. vaginalis* present in 62 of 164 cases of vaginitis (37.8 per cent). Stewart stated: "In the pure single infection cases, *Trichomonas vaginalis* still holds the lead over *H. vaginalis*, but as our diagnostic ability has increased, there seems to be a gradual increase in the proportion of bacterial disease."⁷

Pathogenicity

Difficulty has been encountered in proving by the classic Koch's postulates that *H. vaginalis* is a vaginal pathogen. In our original detailed publication² we reported that 13 patients had been inoculated with pure cultures of *H. vaginalis*, and that 10 of these failed to develop either clinical evidence of the disease or positive cultures. Two patients yielded positive cultures for 2 and 3

months, respectively, but neither developed typical signs of the disease. One patient, however, developed clinical manifestations of the disease, and the organism was recovered in pure culture on one occasion. In the interim, we have inoculated 16 additional patients with pure cultures of *H. vaginalis* without producing the typical clinical signs. It may be postulated that passage of *H. vaginalis* through the artificial media currently employed alters its inherent infectivity.

Other evidence of pathogenicity has been reported in our previous publication.² Fifteen normal patients with Grade I or Grade II flora were inoculated directly with material from infected vaginas, and 11 of these women developed classic laboratory and clinical signs of *H. vaginalis* vaginitis.

We have observed, for a period of 3 to 6 years, 7 untreated patients with naturally acquired infections who have shown no major variations in the clinical or bacteriological patterns of their *H. vaginalis* vaginitis.

To date, 6 patients have been observed to develop *H. vaginalis* vaginitis immediately after marriage. Premaritally, these patients exhibited normal vaginas and yielded lactobacilli on smear and culture.

We have cultured the urethras of 101 husbands of infected wives; of these, 91 yielded *H. vaginalis* as the predominant organism. In contrast, urethral cultures were obtained from 38 male medical students, with the organism being isolated from only one of them. It has been a repeated observation that treated patients develop recurrences if their husbands are not treated simultaneously with appropriate antibiotics.

Ray and Maughan stated "... *H. vaginalis* has been accepted here as the etiological agent of a specific disease entity."⁴ While Amies and Jones⁵ and Lutz *et al.*³ have independently recognized *H. vaginalis* as an etiological agent in nonspecific vaginitis, they do not present detailed clinical descriptions. Brewer and his associates have stated: "Clinical evidence of pathogenicity is suggested by the frequency with which this organism is the predominant one in the vaginal flora of patients with bacterial vaginitis and by the fact . . . that the leukorrhea and vaginitis disappear when *H. vaginalis* disappears from the flora."⁶ They stated further: "In our study the flora varied considerably in individual patients over the year and one-half that cultures were made. No one organism was consistently found in association with *H. vaginalis* which might be considered capable of producing infection and leukorrhea. These bacteria appeared and disappeared from the flora without any rhyme or reason as far as leukorrhea was concerned. As a cause of leukorrhea in the patients studied the bacteria other than *H. vaginalis* were obviously unrelated to vaginitis."⁶ The opinions expressed by Brewer and his associates from their own clinical and laboratory experience coincide exactly with our own. Whenever *H. vaginalis* is exhibited in vaginitis, the clinical pattern of the disease is identical, whether the patient yields the organism in pure or in mixed bacterial culture. After *H. vaginalis* is eradicated, the clinical signs of the disease disappear, with immediate restoration of normal vaginal secretions (TABLE 1).

The fact that pure cultures of *H. vaginalis* are not obtained on each attempt furnishes no valid basis upon which to question the etiological relationship of this organism to the disease entity. The finding of 181 pure cultures in the 940 sequential tests of this study seems to be of greater significance. It is common

for transient organisms to appear and to disappear within a period of a few days, while *H. vaginalis* persists. The number of these occasional organisms are relatively inconsequential, and their occurrence or disappearance fails to alter the clinical pattern of the disease entity. The transient bacteria sporadically associated with *H. vaginalis* also occur in association with lactobacilli in normal vaginas and in association with trichomonads in trichomoniasis.

Miscellaneous Bacterial Infections

During the period 1953 through 1959 in the private office series, we have encountered only 29 patients with bacterial vaginitis that could not be attributed to *H. vaginalis*. Of these, 16 were attributed to the following organisms: *Staphylococcus aureus*, 3; beta hemolytic streptococci, 3; *Proteus* sp., 2; *Pseudomonas aeruginosa*, 1; alpha hemolytic streptococci, 2; *Escherichia coli*, 2; *Neisseria gonorrhoeae*, 2; and *Hemophilus ducreyi*, 1. Other than Ducrey bacilli and gonococci, these species are not assumed to be specific vaginal pathogens. Thirteen patients, although diagnosed clinically as having bacterial vaginitis,

TABLE 1
CHANGES THAT OCCUR CONSISTENTLY WITH THE ERADICATION OF *H. VAGINALIS*

	<i>H. vaginalis</i> discharge	Normal secretions
Color	Gray	→ White or slate
Malodor	+	→ 0
Frothiness	0+	→ 0
Consistency	Homogeneous	→ Curdy
pH	4.8-5.5	→ 3.8-4.5
"Clue cells"	+	→ 0
Bacteria	<i>H. vaginalis</i>	→ Lactobacilli

could not be classified etiologically. The majority of the identified and unidentified bacterial agents in this group of 29 miscellaneous bacterial vaginitides represented temporary imbalances in flora, secondary to extraneous causes or to altered physiology. The *Pseudomonas*, *Proteus*, and *Staphylococcus aureus* infections had, in most instances, followed antibiotic therapy. Of this group of 29 patients, only those with beta hemolytic streptococcal infections displayed a uniform clinical pattern.

Comments

There are some statistical differences in this report as compared to an earlier report by us and reports of other investigators. However, the present study is based on a large number of individual observations in a group of patients with *H. vaginalis* vaginitis as a single infection; patients yielding trichomonads or *Candida* were excluded.

Occasional difficulty may arise in determining whether or not a vaginitis exists in a particular patient. However, a woman in the childbearing age with normal estrogens who displays a vaginal discharge not attributable to cervicitis, which is abnormal in color, odor, consistency, and pH, and which shows a Grade III flora ordinarily should be considered to have vaginal pathogens and vaginitis.

The term vaginitis today has a broad interpretation that includes patients other than those with gross and microscopic tissue changes. While *H. vaginalis* is associated with an abnormal and malodorous discharge and is an infectious entity, it is only occasionally associated with gross and microscopic morphologic abnormalities. Argument may be advanced against calling this infection an inflammatory disease as the term vaginitis implies; however, it must continue to be included in this category until such time as a more suitable term has been proposed and accepted.

To determine accurately the true clinical and laboratory pattern of a vaginal infection it becomes necessary to exclude patients who have marked cervicitis, estrogen deficiency, or bleeding; patients who have recently douched, had coitus, or used vaginal medicaments and contraceptives; and patients who yield a second vaginal pathogen. Except for special projects, institutionalized patients and children are poor subjects for studying bacterial vaginitis. For basic research, charity patients pose many uncontrollable problems. To attempt definitive cultures on vaginal material from charity patients can be a frustrating experience because the vaginas of many of these people are teeming with a large variety of microbes. Overgrowth of a specific pathogen by numerous and more hardy contaminating organisms is a common occurrence. If there is to be an agreement between independent investigators, a strict categorizing of patient material and standardization of methods becomes essential.

On the basis of our findings, together with those of other investigators, it seems evident that the riddle of so-called "nonspecific" vaginitis has been largely solved. Also, perhaps many of the leukorrheas previously attributed to such dubious causes as vitamin deficiency, pelvic congestion, psychoneurosis, and allergy are in reality caused by *H. vaginalis* infections.

Conclusions

There exists a specific infectious disease entity of the vagina that is attributable to a small Gram-negative bacillus of the genus *Hemophilus*.

The disease entity accounts for over 90 per cent of vaginitides previously classified as "nonspecific."

The disease demonstrates a uniform clinical and laboratory pattern.

The disease is easily diagnosed by correlating clinical findings with wet-mount and stained-smear findings.

The disease is communicable by sexual contact.

Eradication of the small Gram-negative bacillus is followed by disappearance of the disease.

H. vaginalis is the only organism consistently present in the described entity.

The term "nonspecific" vaginitis is a misnomer and should be discarded.

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HEMOPHILUS VAGINALIS AND NONSPECIFIC VAGINITIS

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In the past few years several articles on nonspecific vaginitis have appeared in the medical literature. Although Leopold¹ first described *Hemophilus vaginalis* in 1953, Gardner and Dukes,² in 1955, incriminated this microorganism as a single etiological agent in so-called nonspecific vaginal infections. Other workers^{4, 5} have tried to duplicate the work of Gardner and Dukes, with more or less success.

This study was undertaken to attempt to clarify the role, if any, of this small Gram-negative anaerobic microorganism as the causative agent in nonspecific vaginitis. As to the pathogenicity of the microorganism, a controversy still exists. Some authors⁶ deny its existence as a single entity and doubt its capabilities to produce disease, while others^{2, 3} have tried to describe and classify it. The laboratory methods I employed in this study duplicated those used by Gardner and Dukes in detecting the presence or absence of *H. vaginalis*. I have also tried to determine from the data obtained, whether *H. vaginalis*, when found, was the sole infecting microorganism responsible for the signs and symptoms of nonspecific vaginitis.

In this study I have used a new form of Triple-Sulfa vaginal therapy; however, in this paper therapy is mentioned only to illustrate the behavior of *H. vaginalis* as compared to other microorganisms.

Material and Methods

One hundred and twenty-seven patients were selected from the antepartum and postpartum clinics, as well as from the gynecological outpatient clinic of the Methodist Hospital.

All of these patients selected complained of pruritus, burning, and vaginal discharge. All had one or more of the following signs: erosion of the cervix, endocervicitis, vaginitis, or evidence of pruritus and vaginal discharge. Pathology of the uterine corpus or adnexa was ruled out by a complete pelvic examination.

A wet-mount preparation was performed on all possible candidates for this study, and those showing signs of trichomonal infection were rejected. Those patients who qualified for this study were subjected to the following bacteriological examination of their vaginal secretions. A smear for Gram staining was obtained from the posterior fornix. Two separate swabs were used to collect material from the same area for culturing and were placed in a sterile glass test tube. This material was then processed in the hospital laboratory within a period of 2 hours. Specimens were transferred from these tubes to Brewer's thioglycolate broth without indicator. Two blood agar plates were properly streaked; one was placed for aerobic incubation and the other was placed in a CO₂ candle jar. All cultures were incubated at 37° C. for 48 hours. Secretions from the lateral vaginal wall or swabbings of suspicious areas, if present, were obtained on another applicator. This was streaked on a vial of Nickerson's medium to rule out the presence or absence of the genus *Candida*.

Smears from material obtained were examined for bacterial flora by Gram's stain before and after culturing. Small Gram-negative anaerobic bacilli not found in routine aerobic cultures were considered *H. vaginalis*, as they fulfilled the criteria described by Gardner and Dukes. Staphylococci were classified according to their pigment formation on nutrient agar, streptococci according to their reaction on blood agar or growth in Brewer's media. The aerobic bacilli were streaked on Endo's media for preliminary identification, and were processed further if necessary.

After the above-described material was obtained for laboratory investigation, each patient was given sufficient Triple-Sulfa vaginal tablets to last for a 10-day period. On the follow-up visit 14 days after the first smears and cultures were obtained, each patient was subjected to the same examination, and material was obtained from the vagina in exactly the same manner as on the previous visit. The laboratory procedure was also identical to the one described above.

TABLE 1

	HVb, HVa	HVb, noHVa	noHVb, HVa	noHVb, noHVa	Total
Improved.....	4	11	8	49	72
Not improved.....	1	2	4	11	18
	5	13	12	60	90

Key: HVb, patients had *H. vaginalis* before treatment; HVa, patients had *H. vaginalis* after treatment; noHVb, patients had no *H. vaginalis* before treatment; noHVa, patients had no *H. vaginalis* after treatment.

Note: Only 90 patients were considered in the statistical analysis, as 37 patients failed to return for a follow-up visit after treatment.

Results

In a total of 218 cultures a variety of microorganisms was found to be present in the vaginal flora of all patients selected for this investigation. These bacteria were: *Staphylococcus albus*, *Staphylococcus aureus*, *Streptococcus* (anaerobic), nonhemolytic *Streptococcus*, alpha- and beta-hemolytic *Streptococcus*, diphtheroids, *Escherichia coli*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*.

One or several of these organisms appeared in the cultures obtained. A small Gram-negative bacillus conforming to the description of *H. vaginalis* was found in 30 cultures. In not one instance were these anaerobic bacilli the only bacteria present in the vagina; consequently, they were not isolated in a pure culture. The genus *Candida* was found in 31 cultures, and also was always in association with some other microorganisms.

Statistical Analysis

Classification of patients according to incidence of *H. vaginalis* before and after treatment is shown in TABLE 1. Application of a Chi-square test of significance shows that the classification in TABLE 1 of HV variable versus improvement is independent. In other words, there is no relation between pa-

tient improvement or lack of improvement and the presence or absence of *H. vaginalis*.

Computation

$$\begin{array}{cccc}
 \frac{4(4)}{1(1)} & \frac{11(10.4)}{2(2.6)} & \frac{8(9.6)}{4(2.4)} & \frac{49(48)}{11(12)} \\
 x^2 = 0 + \frac{(11 - 10.4)^2}{10.4} + \frac{(8 - 9.6)^2}{9.6} + \frac{(49 - 48)^2}{48} + 0 \\
 + \frac{(2 - 2.6)^2}{2.6} + \frac{(4 - 2.4)^2}{2.4} + \frac{(11 - 12)^2}{12} = 1.58
 \end{array}$$

Value of x^2 for significance at the 0.05 level, with $n = 2$; degrees of freedom = 5.99. Since 1.58 is smaller than 5.99, classification shows independence between the two variables.

The largest difference in proportion is observed in group noHVb, HVa.

$p_1 = \frac{8}{72} = 0.11$, proportion of improved patients not having *H. vaginalis* before treatment and having *H. vaginalis* after treatment.

$p_2 = \frac{4}{18} = 0.22$, proportion of unimproved patients in same category.

The difference in these two proportions is not significant.

Computation

$$t = \frac{p_1 - p_2}{\sqrt{\frac{pq}{n_1} + \frac{pq}{n_2}}} = \frac{0.11 - 0.22}{\sqrt{\frac{(0.13)(0.87)}{72} - \frac{(0.13)(0.87)}{18}}} = -1.2$$

$p = \frac{8 + 4}{90} = 0.13$, value of t for significance at the 0.05 level = -1.96

The difference is not significant.

The type of microorganisms present before and after treatment in improved and unimproved patients varies. The presence or absence of certain types of microorganisms before and after treatment seemed to occur in a random fashion, and was not related to the improvement or lack of improvement of the symptoms.

Discussion

Nonspecific vaginitis may be caused by a variety of microorganisms, as the results of this study indicate. The mere fact that any one or any number of organisms has been cultured from a diseased vagina does not mean that those organisms are all responsible for the vaginitis. This point is illustrated by those cases in which I obtained positive cultures for one or several microorganisms before as well as after treatment, although the subjective and objective signs and symptoms have cleared completely after therapy.

H. vaginalis never could be obtained in pure culture, as it always was associated with some other microorganism. It was found to be present after treatment in 12 cases where it could not be demonstrated prior to therapy. In 8 of these cases the symptomatic and clinical response to therapy was very satisfactory. In 3 cases *H. vaginalis* was present before and after treatment, although these patients all responded to therapy. The presence of these organisms in the vagina does not appear to be more significant than the presence of the numerous other bacteria observed in nonspecific vaginitis. Our data do not substantiate the role of these organisms as an etiological agent causing nonspecific vaginitis.

As the response to therapy had nothing to do with the presence of a specific organism, multiplicity of any one microorganism, or with patterns of grouping, I believe that in nonspecific vaginitis there is no need to make cultures in order properly to treat the patient. In an attempt to identify the microorganisms present in certain pathological conditions cultures of the vaginal flora should be reserved for research purposes only, as they do not give us any practical information.

Summary

The results of this investigation clearly demonstrate the presence of a Gram-negative bacillus conforming to the description of *H. vaginalis* in the vaginal flora. This organism was never isolated in pure culture, but always was found to be associated with one or more bacteria. No evidence was found to suggest that *H. vaginalis* is the etiological agent in nonspecific vaginitis.

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Part IV. The Vagina in Reproduction: Miscellaneous

THE VAGINA AND INFERTILITY

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The vagina is a reproductive organ of major importance, but many aspects of its relationship to the procreative function are not clear. Its role in coitus is obvious, as well as its participation in parturition, but there are other areas of vaginal function where considerable gaps in our knowledge exist. Previous papers in this monograph have treated a number of these very competently; I shall limit my paper specifically to the relation of the vagina and its secretions to the semen.

Since the ability to reproduce is the most vital aspect of human physiology, it might be expected that, under normal circumstances, the vagina and its activities would create an environment completely favorable to the occurrence of pregnancy. However, from a relatively superficial standpoint, it would appear that this is not entirely true, and that there are certain inconsistencies in the relationship of the vagina to reproduction. (The word superficial is emphasized because it is entirely possible that, with increased study, some of these inconsistencies might be completely consistent as far as favoring reproduction is concerned.)

In the first place, we can refer very quickly to the obvious facts that spermatozoa maintain their motility best in a slightly alkaline pH, yet the vaginal secretions normally are fairly strongly acid. How can this be reconciled with the fact that humans are given such a wonderfully intricate and generally favorable set of physiological circumstances to favor the sperm reaching the egg?

Second, why do the vaginal walls approximate themselves so closely after intercourse that, in some instances of infertility, semen leakage appears to play at least some role? The posing of this question does not ignore the fact that a large, hollow vagina would be poorly effective during coitus.

Another normal situation that appears strange is the presence of a cul-de-sac where a seminal pool, from which spermatozoa would not have easy access to the cervix, might form. Also, is not the normal close proximity of the vaginal orifice to the anus and its bacterial flora something that can have an anti-reproductive potential?

Later in this paper it may be possible to speculate briefly about a few of these questions, but I shall proceed immediately to a discussion of the specific matter of vaginal-spermatozoal relations.

In order to evaluate the findings in the vagina itself, these necessarily must be correlated with information obtained from semen analysis and cervical mucus studies. While the latter secretions are, of course, not vaginal, their close proximity to the vaginal cavity requires their inclusion in this discussion; furthermore, cervical mucus and secretions from elsewhere in the genital tract

are often contaminants of vaginal secretions. Conversely, vaginal secretions very often contaminate the more external portions of the cervical mucus.

For many years, physicians interested in infertility have been accustomed to performing postcoital studies known as Sims, Sims-Huhner, or Huhner tests. In general, these tests involve obtaining and observing in the laboratory secretions obtained from the vagina and cervix some time after intercourse. More recently, with the increased use of semen analysis, the vaginal portion of the test is sometimes omitted, although it still has considerable significance in some instances.

This paper summarizes postcoital findings in an infertility practice among several categories of patients. The latter include: (1) patients showing no evidence of vaginal pathology or seminal inadequacy; (2) patients showing recent or current signs of trichomonal infection, but where the husbands' semen is relatively normal; (3) those having so-called nonspecific vaginitis and normal semen; (4) patients showing moniliasis in the presence of normal semen; and (5) instances where there are low semen volumes but normal spermatozoal concentration, morphology, and motility. Patients with oligospermic husbands are excluded from this report.

Procedure of Study

Patients were requested to report for examination at varying hours following coitus, and samples of secretions were taken from the vagina and cervix. In order to be able to compare findings more readily, the tests herein reported were made at similar cycle times in relation to ovulation, except where otherwise indicated. For practical purposes, the time of ovulation was presumed to be approximately fourteen days before the next anticipated menstrual period. No specific attempt was made to have all patients in this group come to the office very promptly after coitus; rather, it seemed preferable to employ observations at various times. Relative to proximity of the tests to ovulation, it is, of course, well established that the postcoital findings in cervical mucus are very strongly correlated with the phase of the cycle in which the test is done, but very little has been reported in the past concerning variations in vaginal findings related to time of the menstrual cycle. Since data concerning the latter point are not included herein, it may be mentioned in passing that there appears to be some cyclic correlation, but probably largely because the cervical mucus is so much more profuse at ovulation that the mucorrhea at times favorably contaminates the vagina.

In obtaining secretions for examination, a very simple procedure is used. A nonlubricated speculum is inserted in the vagina, and long, narrow 3-cc. syringes are employed by suction to aspirate material from various sections of the vagina. In instances where secretions are very scanty, material may at times be obtained from the blades of the speculum on its removal.

Cervical mucus is obtained from the patient by first wiping the cervix with cotton about the external os to remove as much vaginal contamination as possible and then inserting the tip of the syringe through the os and applying suction. Ordinarily when this is done near ovulation at least one quarter of a cubic centimeter of mucus, and very often much more, can be obtained.

Uniform-sized drops of vaginal and cervical material are placed on glass slides in amounts that spread over the area of a thin cover slip when the latter is placed lightly on the slide. (These notations are included herein to indicate that attempts were made to obtain drops of fairly uniform thickness for comparative microscopic evaluation.) Separate portions of secretion are placed on other slides for pH determination and observations of spinnbarkeit, fernlike crystallization, and consistency.

Observations

TABLE 1 summarizes the postcoital observations in a group of patients where the husbands' semen previously was found to be normal and there was no evidence of vaginal or cervical pathology. As may be noted, there was considerable individual variation. Shortly after intercourse it is not uncommon

TABLE 1
POSTCOITAL STUDIES OF A GROUP OF INFERTILE WOMEN HAVING NORMAL VAGINAL FINDINGS
(HUSBANDS' SEMEN NORMAL)

PT.	Cycle D/L	Hours P-C	Vagina			Cervix		
			Sp HPF	Mot. Percent- ages & G.	pH	Sp HPF	Mot. Percent- ages & G.	pH
1	16/30	4	30/1	20 C	6.0	20/1	60 A	7.5
2	14/30	14	4/1	0	4.5	10/1	40 B	8.0
3	15/29	2	60/1	70 B	7.5	60/1	80 A	8.0
4	19/32	16	1/1	0	4.5	15/1	60 B	8.0
5	9/24	12	6/1	0	4.5	25/1	40 C	8.0
6	20/?	134	0	—	4.5	1/5	10 C	8.5
7	17/30	11	4/1	0	4.0	7/1	20 B	7.5
8	14/28	3	75/1	40 B	7.0	40/1	70 B	7.5
9	11/25	16	0	—	4.5	4/1	50 B	7.5
10	15/28	36	0	—	4.5	0	—	7.5

to find some type of seminal pool in which the concentration of spermatozoa approximates that of the semen itself. On the other hand, as the interval of time between coitus and examination increases, as may be expected, there is a fairly rapid change, particularly in sperm motility. The cervical mucus sperm concentration (and motility) may also show considerable variation within the normal control group. Of particular note is the inclusion of one case where spermatozoal motility was observed in the cervical mucus 134 hours after intercourse. Reports have indicated generally that 72 hours is the motility span of spermatozoa in the cervical secretions, but this is certainly not a specific limit. In fact, in one instance not reported in this particular series, I have observed motile spermatozoa in the cervical mucus 6½ days after intercourse. (I have no reason to doubt the accuracy of the patient's memory.) There is one important situation that apparently exists when cervical sperm motility of unusually long duration is observed. It occurs among women who do not have normal cycles, but who have a state of persistent estrinism in which the

mucus remains thin, translucent, acellular, and shows considerable spinnbarkeit for many consecutive days. This type of favorable mucus is ordinarily found relatively briefly in the normal cycle, for the period of significantly marked estrogenicity is displaced relatively rapidly by luteal influence. In anovulatory cases, the luteal effect on cervical mucus is not present, or very insignificant; hence the persistently favorable secretions. In relation to these particular secretions, one must speculate on the identity of the mucus from the standpoint of whether a "batch" of mucus stays in the cervical canal for a considerable length of time or whether there is continuing active formation of new mucus, with the old mucus draining off and carrying with it some spermatozoa. This is a rather vague consideration in evaluating cervical mucus findings, falling into a similar class with the often-referred-to suggestion that the finding of few sperm in cervical mucus several hours after coitus simply means that most of them have progressed to the Fallopian tubes where they belong.

TABLE 2

AVERAGE POSTCOITAL FINDINGS IN A GROUP OF INFERTILE WOMEN HAVING *TRICHOMONAS VAGINALIS* INFECTION (A) COMPARED WITH GROUP HAVING NO INFECTION (B)

	Number of patients	Average hours P-C	Vagina			Cervix		
			Sp HPF	Mot. Percent-ages & G.	pH	Sp HPF	Mot. Percent-ages & G.	pH
(A)	14	11	1/1	0	>5.5	8/1	<40	8.0
(B)	5	14	3/1	0	<4.5	12/1	>40	7.5

TABLE 2 presents a summary of findings among women with trichomonad infection in instances where the semen is normal, and compares the average findings with those in the TABLE 1 group done at similar time intervals. All tests were not performed when there was marked leukorrhea, but the patients in this group very recently or currently had shown trichomonads in the secretions. As may be noted, the average pH of such secretions is higher than in normal secretions, and sperm concentration is lower than that found at similar time intervals in normal cases. One reason for the latter condition may be increased dilution of sperm concentration by leukorrheic secretions. Cervical mucus findings, while not as satisfactory as in normal instances, are not markedly dissimilar.

TABLE 3 summarizes the findings noted when nonspecific vaginitis is present. Some of the cases included actually may be instances of *Trichomonas vaginalis* infection, but the organism was not demonstrated. As may be observed there are some deviations from average normal findings, but these differences are not striking.

When monilial infection is present there is increased vaginal acidity, but apparently this is not sufficiently marked greatly to alter the cervical mucus findings, as illustrated in TABLE 4.

When semen volume is unusually small, despite the presence of normal sperm

concentration, motility and morphology, postcoital findings are usually poor in the infertile women in this series. Sperm migration into the cervix is markedly reduced, compared to normal, as shown in TABLE 5. It is my impres-

TABLE 3

AVERAGE POSTCOITAL FINDINGS IN A GROUP OF INFERTILE WOMEN HAVING NONSPECIFIC VAGINITIS (A) COMPARED WITH GROUP HAVING NO INFECTION (B)

	Number of patients	Average hours P-C	Vagina			Cervix		
			Sp HPF	Mot. Percent-ages & G.	pH	Sp HPF	Mot. Percent-ages & G.	pH
(A)	6	10	1/1	0	>5.0	6/1	50	7.5
(B)	5	14	3/1	0	<4.5	12/1	>40	>7.5

TABLE 4

AVERAGE POSTCOITAL FINDINGS IN A GROUP OF INFERTILE WOMEN HAVING *CANDIDA* INFECTION (A) COMPARED WITH GROUP HAVING NO INFECTION (B)

	Number of patients	Average hours P-C	Vagina			Cervix		
			Sp HPF	Mot. Percent-ages & G.	pH	Sp HPF	Mot. Percent-ages & G.	pH
(A)	7	10	1/5	0	<4.0	8/1	30	7.5
(B)	5	14	3/1	0	<4.5	12/1	>40	>7.5

TABLE 5

AVERAGE POSTCOITAL FINDINGS IN A GROUP OF INFERTILE WOMEN HAVING NORMAL VAGINAL CONDITIONS WHOSE HUSBANDS' SEMEN SPECIMENS SHOW CONSISTENTLY LOW VOLUMES (1 CC. OR LESS)

	Number of patients	Average hours P-C	Vagina			Cervix		
			Sp HPF	Mot. Percent-ages & G.	pH	Sp HPF	Mot. Percent-ages & G.	pH
(A)	8	12	1/4	0	<4.5	1/1	<40	7.5
(B)	5	14	3/1	0	<4.5	12/1	>40	>7.5

sion that small volumes of semen do not possess adequate buffering capacity or adequate physical neutralizing to counteract vaginal acidity, and that therefore the spermatozoa are immobilized relatively promptly. In such instances, then, normal vaginal acidity constitutes a barrier, relatively speaking, to conception.

Discussion

As previously stated, one of the most obvious questions relative to the vagina and infertility is: why do normal vaginal secretions have a pH range unfavorable to spermatozoa? Since the vagina may be considered at least a temporary repository for spermatozoa, it might be expected that a favorable environment would be encountered there. It may be conjectured, on the other hand, that sperm are not intended to collect in the vagina and that the spermatozoa destined to be useful for fertilization are supposed to gain immediate access to the cervix. Theoretically, these sperm may be ejaculated directly on the cervix; it can be also speculated that the early coagulated form of semen is for the purpose of helping it cling to the cervix and mix with its mucus. Support for this latter theory of relatively direct transmission to the cervix would, of course, come from those who claim that there is so-called "insuck" on the part of the uterus during coitus.

On the other hand, it would be more reasonable to find an explanation for vaginal acidity that fits in with the possible functions of this acidity. Obviously, an acid vaginal environment favors the maintenance of a healthy mucosa and helps prevent infection from a variety of organisms, but one might also argue that vaginal acidity helps promote fertility by a sort of reverse chemotaxis in which the spermatozoa are drawn away from it toward the more favorable pH of the cervix. (This implies, of course, that the low vaginal pH would not act deleteriously too promptly on the sperm to prevent sperm migration.)

On this subject another factor requires consideration, and that is the nature of the so-called lubricating secretions that appear during coitus. I am unaware of detailed prior studies of the chemical nature of these substances, per se, since to obtain them with no admixture of semen is obviously a problem. It may well be that these secretions actually help to counteract acidity during intercourse, and that a safeguard is therefore present.

As far as vaginal infections are concerned, from the purely laboratory standpoint these apparently do not exert a harmful effect upon fertility. Clinically, they undoubtedly temporarily reduce fertility in that often coitus is apt to be avoided. The burning, itching, and general discomfort may also extend to dyspareunia; even the leukorrhea itself also may contribute toward less frequent or less effective coitus. Furthermore, certain of the medications used in therapy, if employed near ovulation, may prove hostile toward spermatozoa. However, if the broad problem of infertility is considered, it is unlikely that any significant percentage of patients with true, long-standing fertility problems will have their barrenness caused by vaginal infections of the type discussed in this report.

There are other ways in which the vagina may affect fertility that are not included in this discussion. For example, in a number of instances the cervix, particularly when the uterus is retroverted, will point high in the vaginal vault, and this position may not favor sperm transfer, yet the number of patients in whom this situation occurs with normal transfer makes us wonder about its significance. Also, there are the relatively infrequent instances where the vagina is so long and the cervix so far from the orifice that sperma-

tozoa appear to have trouble reaching the cervix; at least, postcoital tests would so indicate.

It should be emphasized that, as far as secretions are concerned, these discussions have been limited to abnormalities in the vagina. While there seems to be little evidence to incriminate the more common vaginal infections in the infertility problem, this does not hold for the cervix. Many clinicians believe that abnormalities of the cervix and its secretions are quite often responsible for sterility. Whether a substantial number of cervical problems arises from vaginal disorders is another consideration; if we were to agree that they do, then obviously we would have to conclude that the vagina indirectly plays a major role in infertility.

Conclusions

There is a relatively wide range of spermatozoal concentration and motility in the normal vaginal and cervical secretions of women following coitus when the semen is normal.

Vaginal infections that are associated with increased vaginal pH apparently do not alter the postcoital findings to a great degree.

Vaginal infections associated with lowered pH ordinarily do not affect postcoital findings to a significant degree.

When semen volumes are consistently lower than 1 cc., postcoital tests are relatively poor, probably as a result of inadequate seminal buffering capacity.

Vaginal infections causing inflammation and dyspareunia may impair fertility temporarily by reducing the frequency and possibly the effectiveness of intercourse.

THE SEXUAL RESPONSE CYCLE OF THE HUMAN FEMALE: VAGINAL LUBRICATION*

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During the past five years, the anatomical and physiological responses of the human female to effective sexual stimulation have been under evaluation by the Department of Obstetrics and Gynecology, Washington University School of Medicine. Problems of origin, local physiological influence and, ultimately, biochemical definition of the phenomenon of vaginal lubrication become obvious immediate by-products of any primary anatomical investigation. The techniques of colored motion-picture photography have provided definitive analysis of the normal intravaginal anatomical reaction to various phases of the female's sexual response cycle. In addition, over one hundred complete orgasmic cycles have been carried to successful conclusion with the vagina exposed by speculum placement. Direct observation of vaginal lubrication as it develops has been one of the most interesting by-products of the original anatomical investigation. The influence of sexually stimulated vaginal lubrication upon existing base-line vaginal physiology is of present concern to the Sex Research Project that is being conducted at the Washington University School of Medicine.

Any discussion of the lubrication phenomenon necessitates fundamental knowledge of vaginal reaction to effective sexual stimulation, and a description of basic terminology. The human female's sexual response cycle has been arbitrarily divided into 4 individual phases. These phases (FIGURE 1) are, in order, (1) excitement, (2) plateau, (3) orgasm, and (4) resolution. The initial and major production of vaginal lubrication is primarily confined to the first phase (excitement) of the complete cycle of sexual response. Detailed reference to the original gross-anatomy paper¹⁶ has been employed in an effort to provide continuity of anatomical description.

The first vaginal anatomical evidence of an excitement-phase response appears on the walls of the vagina within ten to thirty seconds after initiation of either physical or psychic sexual stimulation. This initial and almost immediate response to sexual stimulation is the phenomenon of vaginal lubrication. In the past, there have been innumerable efforts to describe sources of the vaginal lubrication that the human female develops as the result of mounting sex tension. Over the years, many authors have regarded the cervix as the main source of vaginal lubrication.^{1, 3, 5, 7, 9, 13, 17, 19, 28, 30, 32, 33} Bartholin's glands^{5, 12, 18, 24, 29, 32, 33, 36} have also been allotted a major role in the production of vaginal lubrication. Actually, neither the healthy cervix nor Bartholin's glands make any essential contribution to the development of vaginal lubrication.

As physiological response to mounting sexual tension develops, a sweating reaction may be observed on the mucosal surface of the vaginal walls. In-

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dividual droplets of a lubricating material suddenly appear scattered over the rugal folds of the normal vaginal architecture. These individual droplets present a picture somewhat akin to that of a perspiration-beaded forehead. With continued increase of sexual tension the droplets coalesce to form a smooth, glistening coating for the entire vaginal wall. This sweating reaction progresses to establish complete vaginal lubrication early in the excitement phase of the human female's sexual response cycle. The lubrication reaction, as it spreads along the vaginal walls, is the first evidence of effective sexual stimulation. As previously stated, this unique sweating reaction may be initiated easily by psychic, as well as physical, sexual stimulation.

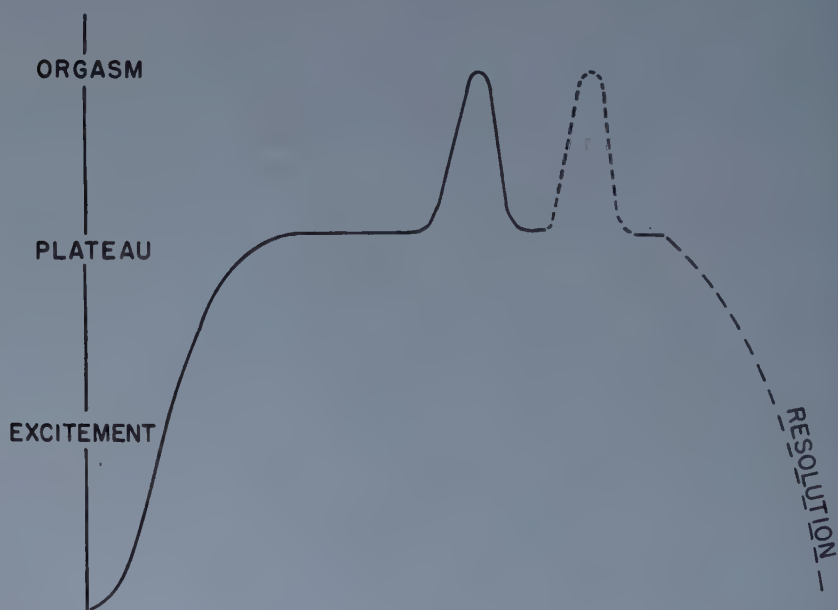


FIGURE 1.

The direct observation of the development of vaginal lubrication has been one of the most interesting aspects of the gross anatomical study of the human female's sexual response cycle. Present etiological conception suggests the possibility that this lubricating material may be the result of a marked dilatation of the venous plexus concentration which encircles the entire barrel of the vagina. The bulbus vestibuli, plexus pudendalis, plexus uterovaginalis and, questionably, the plexus vesicalis, and the plexus hemorrhoidalis externus are all involved to a greater or lesser degree in an immediate and fulminating vasocongestive reaction around the vaginal barrel as sexual tension develops.¹⁶ Thus, as in the male, the human female's primary anatomical response to effective sexual stimulation is a vasocongestive type of reaction. It has long been established that there are no essential glandular elements within the walls of the vagina,¹⁷ yet this lubricating material appears readily (usually in copious amounts), early in the excitement phase of the sexual response cycle.

The vaginal lubrication reaction continues at a greatly reduced (but still significant) rate through the first three phases of the sexual response cycle. However, by far the greatest productivity is confined to early stages of the excitement phase.

During the sexual response cycle, cervical gland secretory activity has been conspicuous by its absence in multiple direct observations of vaginal sexual response. There has been only one observed instance of cervical secretory activity. In this particular situation, the subject lost a mucus plug from the external cervical os under direct observation. She was in the thirteenth day of a regularly-recurring twenty-eight-day menstrual cycle. It was interesting to note that, although the thin, ovulatory-type mucus was certainly extruded from the cervical canal, the reaction occurred late in the plateau phase of the sexual response cycle, long after complete vaginal lubrication had been established.

There are two other fundamental pieces of information that should be considered while on this subject of the lack of any significant cervical, or Bartholin's gland, secretory contribution to the development of vaginal lubrication. First, a pronounced vaginal sweating reaction has been observed repeatedly in a subject who had had a complete hysterectomy and bilateral salpingo-oophorectomy. The ability of this subject to lubricate effectively, even in a surgically castrated state, eliminates the cervix from a functionally significant role as a primary lubrication source. One additional subject, for whom an artificial vagina was surgically created seven years ago, has also been directly visualized during complete sexual response cycles. The artificial vaginal barrel develops effective lubrication, following the pattern of the normal vaginal sweating-reaction response. Additionally, for this subject, there are no Bartholin's glands available to the surgically developed external genitalia. Certainly, there is no cervix in the artificially created vaginal barrel. From the information now available, it may be concluded safely that cervical secretory activity, so frequently described as a constant factor and a functional necessity in the human female's cycle of sexual response, is essentially a nonexistent entity.

It is unequivocally true that Bartholin's glands do respond to effective sexual stimulation in a secretory manner. However, this secretory activity is rarely demonstrable until late in the excitement phase or even early in the plateau level of sexual response.¹⁶ Additionally, the secretory activity usually is confined to a mere droplet or two of a mucoid-appearing material, particularly in nulliparous subjects. The multipara may show a slight increase in the secretory activity of Bartholin's glands, but still does not produce sufficient material to accomplish more than a minimal lubrication of the vaginal fourchette. This secretory activity of Bartholin's glands occurs only well after the entire vaginal barrel has developed a confluent, glistening, surface coating that is the terminal result of the sweating-type reaction of the vagina's primary excitement-phase sexual response.

Methods of Subject Control and Measurement

The influence of the sexually stimulated vaginal lubrication reaction upon pre-existing vaginal pH has been given detailed consideration by the Sex Research Project. Six subjects have been assigned to this particular investigative

effort. Both nulliparous and multiparous individuals have been employed with an age range of from 25 to 35 years. There are 4 normally menstruating individuals, who can successfully achieve orgasm both by manual manipulation and as the result of coital activity. The 2 remaining subjects have been of particular interest to this project. Subject No. 5 is a multiparous female who has undergone a complete hysterectomy and bilateral salpingo-oophorectomy. Thus, sex-steroid levels may be controlled reasonably by careful ingestion techniques with the exception of the obvious, constant adrenal influence. Subject No. 6 is a castrated individual, who has had an artificial vagina surgically developed seven years ago and who is also under carefully controlled, ingested sex-steroid influence.

The 4 regularly menstruating subjects have been followed at twice-weekly intervals, while the 2 castrates have been routinely observed at weekly intervals. The regularly menstruating subjects have been followed repeatedly through experimental control periods of 3 complete menstrual cycles. During the first month's observation, the subject experienced only manipulation-derived orgasms. For the second month's observation, coital orgasm alone was completed. During the third month's observation, the subject's manipulation-derived orgasm was followed shortly by a coitally stimulated orgasmic response. This observation technique has been developed to complete as definitive a picture as possible of the influence of vaginal lubrication upon previously established vaginal pH levels. All subjects spent 1 or 2 months of technique conditioning and of controlled practice measurement before starting on the first of the 3-month periods of recorded observations.

The subjects were protected by condom ejaculate containment for the 3-month control periods during all heterosexual exposures. This technique was demanded to avoid the long-range influence on the established pH of the vagina, which develops from the deposition of the male ejaculate in the vaginal canal. This subject of ejaculate influence on vaginal pH will be discussed in a subsequent paper from the sex research project. All female subjects refrained from the use of douching techniques and no vaginal jelly or suppositories were used at any time during the controlled periods. Also, all subjects were checked at weekly intervals for vaginal pathogens such as *Trichomonas vaginalis* or any of the fungi. No subject disqualification from the control periods for this type of vaginal contamination count has been necessary.

As a preliminary to every individual observation, each subject had a vaginal smear taken and stained after the Shorr technique²⁷ in an attempt to define grossly the influence of the steroid components upon the current phase of the subject's menstrual cycle. A base-line pH measurement was then taken in 5 different sections of the vagina (FIGURE 2). Points *A* and *E* represent areas roughly 1 cm. within the vaginal outlet; *B* and *D* represent areas just lateral to the normally placed, external cervical os (on the lateral vaginal wall); and point *C* is assessed in the depth of the cul-de-sac. Measurements were recorded from these five areas in the vaginal barrel every 2½ min. from the onset of manipulative stimulation, until 4 separate observations had been completed. Thereafter, the individual subject usually was measured every 5 min. until the orgasm phase was experienced. After orgasm, the subject was again checked (during her resolution phase) at 2½-min. intervals for 10 min. and every 5

min. thereafter until stability of the vaginal pH had been satisfactorily demonstrated. This was usually possible within 15 to 20 min. after manipulative orgasm was experienced.

The measurements taken in association with coital orgasm varied, of necessity, in their timing control from the manipulation technique measurements just described. After the original 5-point unstimulated base line had been recorded, the coital unit was separated and pH measurement recorded as soon as the "mounting process" had developed a satisfactory lubrication response from the female partner. After the excitement-phase lubrication observation

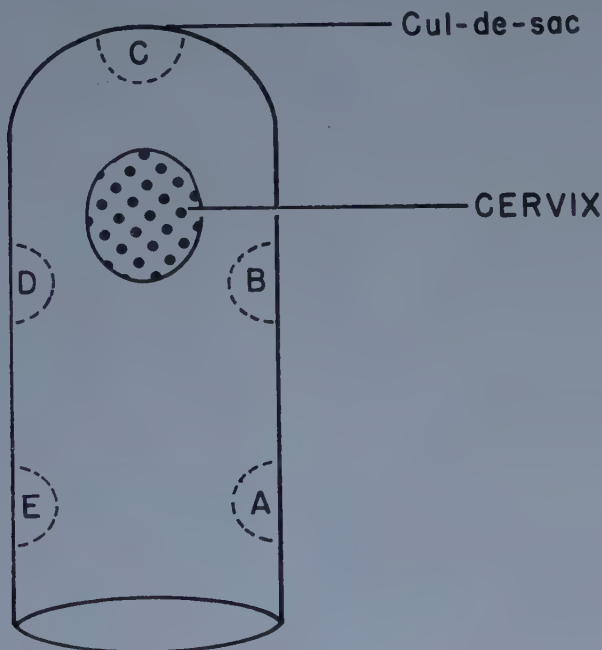


FIGURE 2.

no further pH measurement was obtained until the orgasmic phase had been experienced by the female partner. The orgasmic observations were recorded within 10 to 15 sec. of onset of this most explosive phase of the human female's sexual response cycle. Postcoital resolution-phase measurements followed the timing pattern previously described for the manipulative orgasm, and were continued until stability of the vaginal pH was reasonably demonstrated.

The vaginal pH has been routinely determined with a Beckman Model G pH meter and an 8-in. glass calomel electrode-probe assembly, following the technique popularized by Rakoff *et al.*²² in his modification of the original suggestions of Trussell and MacDougal.³¹ The electrodes were standardized at controlled room temperature, placed in the vagina at the assigned area, and the pH directly determined without speculum separation of the vaginal walls. Intravaginal temperature corrections were not made. However, duplicate

vaginal area measurements could be reproduced with an integrity of about 0.15 pH unit. Importance is attached to differences in pH readings between the established base lines and the experimental values, rather than to the absolute readings.

It should be mentioned that the decision to avoid the separation of the vaginal walls with a speculum (a technique common to previous investigators) was brought about primarily because the speculum technique obviously could not be applied successfully to the measurements obtained during the varying response phases of the coital union. It has subsequently developed, as will be shown, that base-line pH measurements have been recorded normally at significantly lower levels than those described by previous authors. These lower pH readings possibly may represent a truer picture of normal vaginal acidity than has been previously recorded and described.

Results

The influence of vaginal lubrication upon previously established pH levels in the vagina is dependent upon several variants. Of major importance are, in order, (1) severely acid base-line levels of vaginal pH, (2) the amount of lubrication produced by the particular orgasmic cycle under observation, and (3) the length of time consumed by the individual sexual response cycle. If the base-line pH is extremely acid (3.5 to 4.0), usually little, if any, effect upon existing pH levels is created by the vaginal lubrication reaction to manipulation-derived cycles of sexual response. Thus, if a minimal to a moderate amount of lubrication is returned for any particular orgasmic response cycle, there also will be a negligible influence upon any existing severely acid pH of the vagina. Repeated observations also suggest the general rule that the amount of vaginal lubrication usually is increased significantly with coital orgasmic response, as compared to manipulation-derived orgasmic cycles. It is also true that, as a general rule of thumb, the longer the production stimulus for vaginal lubrication is maintained, the more an effect is recorded upon the base-line pH levels of the vagina. Thus, measurements obtained after one-half hour of coital activity, or after multiple manipulation-derived orgasms, would be more likely to have elicited a change even in a severe vaginal pH base line than would a 3- to 5-min. orgasmic cycle, regardless of source of stimulation or amount of lubrication produced.

In an attempt to describe the influence of vaginal lubrication upon existing vaginal pH levels, a cross section of results obtained from 5 of the 6 subjects studied is presented for consideration. To be considered in moderate detail are 3 of the 4 regularly menstruating subjects who are able to achieve an orgasmic experience with relatively equal facility through either automanipulation or coital stimulation. The results obtained with the fourth subject of this group were omitted in the interests of brevity and to avoid unnecessary duplication. The two castrated subjects are minimally recorded more as a means of defining work in progress than as a completed picture ready for objective analysis.

Subject A (32 years old) is considered in TABLES 1 through 3. These 3 tables present results obtained from the same subject on the eleventh, fourteenth, and twenty-fourth days of the first month of a routine 3-month con-

TABLE 1
SUBJECT A: ELEVENTH DAY OF CYCLE (27 DAYS)*

	Min.	A	B	C	D	E
Base line		3.55	3.84	3.94	3.92	3.89
Stimulation begun (mast.)						
Excitement (good lubrication)	2½	3.85	3.87	3.86	3.99	3.89
	5	3.73	4.17	3.86	4.02	3.69
	7½	3.85	3.81	3.84	3.84	3.81
	10	3.79	3.99	4.08	3.96	3.91
Orgasm	19¼	3.90	4.01	4.15	4.01	3.77
Resolution	2½	4.11	3.83	4.18	4.12	3.54
	5	3.89	3.99	3.97	3.97	3.79
	7½	3.82	3.70	3.81	4.08	3.65
	10	3.84	3.92	3.86	4.01	3.70
	15	3.89	4.01	3.99	4.00	3.89

* Last prior orgasm, 5 days (mast.); vaginal smear, preovulatory estrogen peak.

TABLE 2
SUBJECT A: FOURTEENTH DAY OF CYCLE (27 DAYS)*

	Min.	A	B	C	D	E
Base line		3.71	3.75	3.68	3.78	3.59
Stimulation begun (mast.)						
Excitement (good lubrication)	2½	3.72	3.81	3.79	3.68	3.61
	5	3.69	3.89	3.70	3.72	3.59
	7½	3.76	4.09	3.81	3.74	3.75
	10	3.77	3.89	3.83	3.74	3.59
Orgasm	23¼	3.72	3.76	3.69	3.60	3.69
Resolution	2½	3.71	3.71	3.69	3.64	3.80
	5	3.77	3.69	3.62	3.55	3.61
	7½	3.73	3.60	3.77	3.60	3.60
	10	3.75	3.79	3.79	3.68	3.79
	15	3.71	3.62	3.79	3.61	3.71

* Last prior orgasm, 3 days (mast.); vaginal smear, immediate postovulatory.

TABLE 3
SUBJECT A: TWENTY-FOURTH DAY OF CYCLE (27 DAYS)*

	Min.	A	B	C	D	E
Base line		3.62	3.67	3.80	3.99	3.80
Stimulation begun (mast.)						
Excitement (good lubrication)	2½	3.59	3.83	3.90	3.90	3.61
	5	3.71	3.90	3.87	3.91	3.66
	7½	3.71	3.98	3.89	3.94	3.72
	12	3.71	3.84	3.91	3.89	3.88
Orgasm	2½	3.71	3.69	3.85	3.80	3.62
Resolution	5	3.71	3.65	3.91	3.71	3.72
	7½	3.78	3.64	3.90	3.89	3.78
	10	3.69	3.99	3.77	3.96	4.01
	15	3.71	3.90	3.81	3.99	3.75

* Last prior orgasm, 2 days (coitus-C); vaginal smear, late luteal phase.

trolled observation period. During this first month an orgasmic cycle was stimulated only by automanipulative techniques. The recorded base-line observations are of primary interest. The fifteen base-line vaginal pH measurements were taken (as described) at varying stages of the menstrual cycle (preovulatory, immediate postovulatory, and late luteal phases). Despite obvious humoral variations, not a single measurement reaches the height of 4.0 as a pH base-line measurement. The immediate postovulatory acidity (TABLE 2) is slightly lower than the average of the other two base-line measurements, but does not represent a variant of major significance.

Considered in detail, TABLE 1 (preovulatory estrogen peak) shows no significant increase in pH measurements for areas C, D, and E, and only a small rise for areas A and B on the vaginal wall during the entire cycle of sexual

TABLE 4
SUBJECT B: THIRTEENTH DAY OF CYCLE (29 DAYS)*

	Min.	A	B	C	D	E
Base line		3.80	4.08	3.99	3.95	3.83
Stimulation begun (mast.)						
Excitement (fair lubrication)	2½	4.02	4.16	4.02	3.97	4.00
Orgasm	3	4.08	4.00	4.10	3.93	4.19
Resolution	2½	4.10	4.10	4.06	4.01	3.90
	5	3.91	4.21	4.11	3.80	4.00
	7½	3.89	4.10	4.21	3.88	3.92
	10	3.92	4.25	4.09	3.95	3.98
Stimulation begun (mast.)						
Excitement (good lubrication)	2	4.02	4.20	4.00	3.82	4.08
Orgasm	2¾	3.79	4.31	4.30	3.92	4.10
Resolution	2½	3.90	4.19	4.20	3.73	3.70
	5	3.97	4.35	4.41	3.78	3.79
	7½	3.88	4.38	4.32	3.90	3.79
	10	4.02	4.40	4.15	3.82	3.89

* Last prior orgasm, 4 days (mast.); vaginal smear, preovulatory.

response. This table presents, in essence, the picture of a severe base-line pH of the vagina, little (if any) influenced by the production of a good degree of vaginal lubrication during a completely satisfactory manipulation-derived sexual response cycle.

Essentially, the same lack of influence on severe pre-existing vaginal acidity during the course of a manipulative orgasm is also demonstrated in TABLE 2 (immediate postovulatory), despite the fact that a good lubrication response was obtained. The same observation has been reproduced in TABLE 3 (late luteal phase), again despite good lubrication production. Thus, there are 3 instances of reasonably effective vaginal lubrication that have produced no significant change in the severe acidity of the base-line measurements. In these instances there was also a variant of 12 to 23 min. of maintained lubrication response.

The three tables (TABLES 1 to 3) are also presented as evidence of the severe vaginal acidity that is normally produced by subjects controlled from possible vaginal contamination. It is safe to state that vaginal lubrication produced by automanipulative techniques usually has little influence upon vaginal

acidity recorded below the level of a pH of 4.0 as a base-line measurement unless repetitive complete orgasmic cycles are experienced.

Information recorded from Subject *B* (27 years old) is presented in TABLES 4, 5, 6, taken from an immediate preovulatory and two late luteal phases of 3 successive months in a usual 3-month controlled observation period. Again,

TABLE 5
SUBJECT B: TWENTY-FIFTH DAY OF CYCLE (28 DAYS)*

	Min.	A	B	C	D	E
Base line		3.96	3.80	3.99	3.96	3.61
Stimulation begun (coitus)						
Excitement (very heavy lubrication)	9 $\frac{3}{4}$	4.30	3.93	3.99	4.00	3.64
Orgasm	24 $\frac{1}{4}$	4.20	4.10	4.15	4.10	3.81
Resolution	2 $\frac{1}{2}$	4.48	4.51	3.96	4.01	3.60
	5	4.60	4.60	4.02	3.94	3.67
	7 $\frac{1}{2}$	4.71	4.51	4.22	4.10	3.72
	10	4.83	4.76	4.29	4.16	3.96
	15	4.67	4.60	4.49	4.30	3.96
	20	4.70	4.59	4.40	4.50	3.98

* Last prior orgasm, 3 days (coitus-C); vaginal smear, late luteal phase.

TABLE 6
SUBJECT B: TWENTY-THIRD DAY OF CYCLE (28 DAYS)*

	Min.	A	B	C	D	E
Base line		3.69	3.80	3.96	3.86	3.61
Stimulation begun (mast.)						
Excitement (fair lubrication)	2 $\frac{1}{2}$	3.80	3.91	3.91	3.89	3.60
	5	4.10	3.92	3.79	3.84	3.79
Orgasm	5 $\frac{5}{6}$	3.83	3.95	3.76	3.82	3.81
Resolution	2 $\frac{1}{2}$	4.11	3.91	3.57	3.62	3.69
	5	4.05	3.91	3.73	3.93	3.65
	7 $\frac{1}{2}$	4.18	3.75	3.70	3.97	3.51
	10	3.90	3.82	3.78	3.52	3.59
Stimulation begun (coitus)						
Excitement (good lubrication)	7	4.17	4.07	4.12	4.01	3.84
Orgasm	23 $\frac{2}{3}$	4.04	3.88	3.99	3.75	3.87
Resolution	2 $\frac{1}{2}$	4.24	3.93	4.04	3.70	3.58
	5	4.09	4.09	3.97	3.91	3.60
	7 $\frac{1}{2}$	4.19	4.10	4.00	3.96	3.70
	10	4.10	4.07	4.02	3.96	3.81
	15	4.12	4.05	4.15	3.90	3.80

* Last prior orgasm, 4 days (coitus-C); vaginal smear, late luteal phase.

it will be noted that of the 15 base-line measurements taken on this subject, only 1 measurement (*B* in TABLE 4) was recorded above a 4.0 pH level.

The results obtained by this subject from 2 manipulation-derived orgasmic cycles experienced in relatively rapid succession are noted in TABLE 4 (pre-ovulatory phase). The first measured sexual sequence resulted in no significant rise from the base-line pH reading after a 10-min. resolution-phase observation period conducted subsequent to a moderately severe orgasmic reaction. It

should be noted, however, that only fair lubrication was produced during this particular, relatively brief, orgasmic cycle. The patient was then returned to automanipulative stimulation. A subsequent more severe orgasmic experience (although of only $2\frac{3}{4}$ -min. duration) produced a rise in the vaginal acidity measurement at observation areas *A*, *B*, and *C*, but no significant rise at areas *D* and *E* (the right side of the vagina). As the result of the rapid development of the second cycle of sexual response, continued production of vaginal lubrication was encouraged over an observed period of approximately 30 min. This length of production may well have been responsible for the slowly rising *pH* of the vagina (despite the levels of severe acidity at which the observations were begun) rather than the sole influence of the second orgasmic experience. In other words, this may well be an example of a quantitative rather than a qualitative vaginal lubrication influence.

The same subject is detailed in TABLE 5 (late luteal phase) while responding to a coitally stimulated orgasm during the second month of the observation period. It will be noted that the base-line levels are again within the usual realm of low *pH* normalcy repeatedly obtained from these controlled subjects. A significant rise in *pH* was recorded from all 5 vaginal measuring areas during the 20-min. resolution phase subsequent to the orgasmic experience. A very heavy lubrication reaction was developed by the subject during this particular sexual response cycle. It is worthy of note that the excitement-phase measurement was not recordable until nearly 10 min. after sex play had begun, and that the severe orgasmic experience was obtained only after more than 24 min. of constantly maintained sexual stimulation. Therefore, the lubrication reaction was maintained over a protracted period of approximately 45 min. from the time the initial base line was recorded until the final resolution-phase measurements were made.

Further investigative results obtained from Subject *B* during the third month of the controlled period are recorded in TABLE 6 (late luteal phase). In this situation a manipulative orgasm was followed after a 10-min. resolution phase by immediate restimulation in the form of a coitally produced second orgasmic experience. Again, the original base-line measurements were well below the previously accepted minimum of a 4.0 *pH* level. The response to automanipulation was a strong orgasmic experience within 5 min. and 50 sec. from the onset of stimulation. The subject showed no significant rise in vaginal *pH* over a 10-min. resolution observation period, after only a fair lubrication production. The male partner was introduced after the 10-min. resolution-phase measurements were recorded; immediately sexually stimulative techniques were reinstituted. There was a significant rise in *pH* levels at the excitement-phase recording time of 7 min. from the start of the heterosexual stimulation techniques. Orgasmic phase measurements ($16\frac{1}{2}$ min. after the excitement-phase measurements) show some unusual *pH* trends for this particular subject. Finally, the 15-min. resolution measurements again record a significant rise in basic *pH* for areas *A*, *B*, and *C*, but areas *D* and *E* show no significant *pH* change from the original recorded base line approximately 54 min. previously.

This particular subject has been of constant interest to the sex research project during the past two years. Regardless of the technique of sexual

stimulation employed, it has been infrequent to demonstrate any significant rise in recorded pH levels at areas *D* and *E* (the right side of the vagina). (In fact, TABLE 5 contains one of the few positive results recorded for this subject from the right side of the vagina.) Under direct observation, it has been repeatedly demonstrated that she does not develop as good a lubrication reaction from the right side of the vagina as she does from the left. Most of the individual lubrication droplets which coalesce to form coverage for the entire vaginal barrel appear first on her left side and in the cul-de-sac, with a significant reduction in the degree of the usual sweating reaction on her right side. The inconsistency of lubrication production by this subject is particularly evident in view of the long-range, satisfactory pH rise at areas *D* and *E* (right side of the vagina) in TABLE 5, and the short-term, rapidly reversed rise in pH for the

TABLE 7
SUBJECT C: EIGHTEENTH DAY OF CYCLE (30 DAYS)*

	Min.	A	B	C	D	E
Base line		5.22	4.80	4.71	4.89	5.17
Stimulation begun (mast.)						
Excitement (good lubrication)	2½	5.20	4.73	4.72	4.90	5.10
	5	5.03	4.80	4.78	4.89	5.02
	7½	5.10	4.78	4.75	4.80	4.92
	10	5.00	4.80	4.79	4.78	4.94
Orgasm	17½	4.94	4.82	4.69	4.59	4.72
Resolution	17½	4.88	4.79	4.68	4.58	4.63
	2½	4.89	4.79	4.68	4.45	4.64
	5	4.87	4.70	4.68	4.47	4.53
	7½	4.72	4.69	4.60	4.41	4.51
	10	4.80	4.64	4.46	4.38	4.39
	15	4.71	4.47	4.40	4.28	4.29
	20					

* Last prior orgasm, 1 day (coitus-C); clear water douche, 1 day; vaginal smear, post-ovulatory (recent).

same measurement areas in TABLE 6. We have no explanation at present for this individual anatomical and physiological variation.

The material presented for Subject *C* (28 years old) has been selected to show the influence of vaginal lubrication upon higher base-line pH levels in the vagina, as opposed to the previously recorded, severely acid base-line measurements. Information is also presented in this series to support further the contention that the true pH of the noncontaminated vagina is lower than had been previously considered.

During a training-session month, TABLE 7 (postovulatory) was recorded for this subject just prior to the usual 3-month control period. It will be noted that the vaginal pH runs in the high 4 and low 5 range of base-line observation. The sexual-response cycle detailed in TABLE 7 resulted from a manipulation-derived orgasmic experience during which good vaginal lubrication developed. The actual orgasmic phase of the sexual response cycle was experienced after approximately 18 min. of autostimulation. The orgasmic phase was followed by another 20 min. of resolution-phase observation. It will be noted that in all vaginal measurement areas there was a significant reduction in pH levels

from the original base-line measurements. Also of import is the contamination to normal vaginal acidity exemplified by this subject. Although the last prior sexual response cycle had been experienced the previous evening (condom protection was used), this particular coital exposure was followed by a clear-water douche, which has a significant influence upon vaginal acidity. This residual influence of vaginal douching upon existing normal pH levels will be discussed in a subsequent paper from the Sex Research Project, dealing with the subject of the persistent influence of vaginal pH contaminants.

On the eighteenth day of the next recurring menstrual cycle (the first month of a 3-month control period) the base-line figures, TABLE 8 (postovulatory, recent), are again recorded in the very low range of 3.5 to 3.7 pH units. There is a significant rise in this severe acidity, however, subsequent to the production of an advanced degree of vaginal lubrication by the stimulative activity (coitus) recorded here. The excitement-phase measurement was obtained after 8 min.

TABLE 8
SUBJECT C: EIGHTEENTH DAY OF CYCLE (29 DAYS)*

	Min.	A	B	C	D	E
Base line		3.61	3.58	3.59	3.71	3.50
Stimulation begun (coitus)						
Excitement (very heavy lubrication)	8½	3.88	3.85	3.75	3.62	3.70
Orgasm	18¾	4.19	4.14	3.90	3.72	3.69
Resolution	2½	4.10	4.12	3.87	3.78	3.69
	5	4.00	4.15	3.90	3.89	3.79
	7½	4.09	4.02	3.96	3.89	3.74
	10	4.11	4.03	3.95	3.89	3.72
	15	4.02	4.12	3.89	3.91	3.88

* Last prior orgasm, 4 days (coitus-C); vaginal smear, postovulatory (recent).

of heterosexual stimulation, and shows an early but significant rise in pH levels for all but one of the vaginal areas (area *D*). A severe orgasmic phase was experienced after 18 min. and 40 sec. of continued sexual stimulation. After a 15-min. resolution-phase observation period, a pH plateau was obtained. All terminal figures are recorded at levels significantly above the extremely low pH of the base-line measurements. It is interesting to note that these final measurements would be considered to be in a very low pH range if compared to previously held normal vaginal acidity values of 4.0 to 4.9 pH units. This particular table not only demonstrates the influence of an initial very heavy vaginal lubrication, but shows that the maintained production of heavy lubrication over more than 18 min. of constant effective sexual stimulation also has its neutralizing effect upon the originally recorded, severe base-line measurements.

A single table has been chosen to represent the results developing from a complete surgical castrate, Subject *D* (35 years old). This individual's base-line measurements run within the previously described normal pH (4.5 to 5.0) range. The subject had been on the estrogen replacement level of Premarin (5.0 mg./day) for only 2 weeks. The previous castrate vaginal pH levels

recorded for this individual during practice sessions were in the range of 5.5 to 6.0 pH units, while the subject was supported on levels of 1.25 mg. of Premarin per day. It will be noted in TABLE 9 that only moderate lubrication was produced, although manipulative stimulation was maintained for more than 15 min. There was still no significant alteration in the base-line recordings of vaginal pH after a 20-min. resolution-phase observation, or approximately 35 min. after the initial measurements were recorded. Of some significance is the observation that this patient had coitus 2 days previously without attempted condom control of the ejaculate. The long-continued effect of the male ejaculate on the pH of the vagina will be considered by the sex research project in a subsequent paper on the subject of the persistent influence of vaginal pH contaminants.

TABLE 9
SUBJECT D: CYCLE—COMPLETE SURGICAL CASTRATE*

	Min.	A	B	C	D	E
Base line		4.44	4.50	4.69	4.80	4.99
Stimulation begun (mast.)						
Excitement (moderate lubrication)	2½	4.64	4.51	4.62	4.90	4.89
	5	4.70	4.60	4.72	4.82	4.91
	7½	4.67	4.44	4.73	4.98	4.97
	10	4.68	4.59	4.79	4.86	4.81
Orgasm	15½	4.56	4.54	4.75	4.79	4.90
Resolution	2½	4.70	4.52	4.81	4.81	4.75
	5	4.61	4.48	4.80	4.79	4.69
	10	4.62	4.54	4.59	4.69	4.67
	15	4.44	4.49	4.61	4.67	4.70
	20	4.51	4.49	4.71	4.60	4.80

* Last prior orgasm, 2 days (coitus); vaginal smear, good cornification; medication, Premarin, 5.0 mg. o.d.

A single table of observations is also the only representation of Subject E (33 years old) in this paper. This subject is not only a complete castrate, but also has had an artificial vagina developed surgically. Her last previous orgasm was 3 days prior to observation, and was stimulated by coitus without condom control. The vaginal smear showed poor cornification. Her steroid replacement medication at the time of this observation was only 0.1 mg. of stilbestrol taken daily. It is quite obvious that the base-line pH measurements are essentially on the basic side of the pH range. So much so, in fact, that the Beckman pH meter was restandardized to a pH of 7.0 when this subject was under observation. After a 19-min. automanipulative orgasmic experience with the production of moderate vaginal lubrication and a 20-min. resolution-phase observation, there is a significant recorded (TABLE 10) increase in vaginal acidity to a pH average much below the original level of base-line measurement. The 2 castrate subjects will be presented in more detail in a subsequent publication restricted to problems of their particular response patterns.

The pH measurement of cervical mucus^{4, 26} has been of some interest to gynecologic investigators. With this interest in mind, one research subject has been followed through a complete menstrual cycle with measurements of

the cervical mucus taken in the pattern established for vaginal *pH* determinations. There was no demonstrable influence (upon base-line cervical *pH* levels) exerted by complete sexual response cycles stimulated by automanipulative techniques. As previously stated, there was never any evidence of cervical gland secretory activity during any of the observed sexual response cycles.

Discussion

It is obvious from contemplation of the material presented in TABLES 1 through 10 that there are multiple facets to the problem of production of vaginal lubrication during any sexual response cycle. There are also multiple variants in the influence of the vaginal lubrication produced by sexual stimulation upon previously established levels of vaginal acidity. Under strictly controlled conditions, the previously accepted normal *pH* range (4.0 to 4.9) of

TABLE 10
SUBJECT E: CYCLE—ARTIFICIAL VAGINA, COMPLETE CASTRATE*

	Min.	A	B	C	D	E
Base line		6.52	7.08	7.18	7.30	7.20
Stimulation begun (mast.)						
Excitement (moderate lubrication)	2½	5.21	6.51	6.74	7.00	6.57
	5	5.44	6.79	6.81	6.90	6.80
	7½	5.63	6.56	6.69	6.90	6.30
	10	5.55	6.31	6.52	6.89	6.51
Orgasm	19	5.51	6.48	6.59	6.78	6.20
Resolution	2½	4.98	6.20	6.60	6.73	6.34
	5	4.78	6.05	6.48	6.64	6.28
	7½	4.91	5.92	6.37	6.54	6.09
	10	4.91	5.91	6.29	6.55	6.31
	15	5.09	6.01	6.32	6.49	6.39
	20	5.06	5.90	6.22	6.39	6.10

* Last prior orgasm, 3 days (coitus); vaginal smear, poor cornification; medication, stilbestrol, 0.1 mg. o.d.

vaginal acidity^{6, 10, 14, 15, 20, 23, 31, 35, 37, 38} must be reconsidered in the light of returns from the present investigations. During training periods, when intercourse without condom ejaculate control, the use of diaphragms and jelly, and douching techniques was not prohibited, the measurable vaginal *pH* levels ranged within the previously accepted normal of 4.0 to 4.9 *pH* units. During the 3-month control periods, however, when the same subjects were denied the use of any vaginal contamination technique other than condom control of the male ejaculate, the *pH* ranged constantly from a low of 3.5 to a high of 4.2 (with rare occasions recorded above the 4.0 level) for literally hundreds of controlled base-line measurements. These lower *pH* levels were constantly recorded other than during a 48-hour period, just after obvious cessation of menstrual flow. At these times the *pH* of the vagina usually was recorded within the 4.0 to 4.9 range. It was also frequently true that within a day or two before the onset of an expected menstrual period, *pH* ranges from 3.9 to 4.2 were observed, as opposed to the 3.5 to 3.9 *pH* levels constantly recorded for the remainder of any normal menstrual cycle. These minor humoral

variations in recorded vaginal acidity are in complete agreement with the original conceptions of Rakoff²³ and Lang *et al.*¹⁵ It is also obvious that this lends support to the experimental animal results that have paralleled the human female's laboratory evaluation since 1936.^{2, 8, 11, 34}

It is apparent that, at these levels of severe acidity (3.5 to 3.9), there is little or no consistent influence upon the established pH of the vagina from the production of vaginal lubrication, particularly that amount produced by any single automanipulative experience. As a general rule, the amount of lubrication produced during manipulation-induced orgasmic cycles is not equal to that produced by the more usual coitus-stimulated sexual response cycles. Occasionally, a significant rise in the severe base-line pH of the vagina has been recorded subsequent to repetitive manipulative orgasmic cycles, particularly if an effective lubrication reaction has been produced and maintained for an unusual length of time (TABLE 4). In this situation, the production of vaginal lubrication may have to be maintained over periods ranging from $\frac{1}{2}$ to $\frac{3}{4}$ hour, to have any significant effect upon the controlled base-line readings. The effect on vaginal acidity of a manipulative orgasm seldom lasts longer than from 2 to 4 hours. By this time the originally recorded base-line levels of acidity usually have returned to the noncontaminated vagina.

An orgasmic phase successfully produced by coitus usually promotes more effective vaginal lubrication and maintains this production over a longer period of time. Obviously, there is usually a more even distribution of the lubricating material throughout the entire vaginal barrel as the result of the mechanical action of the coital partners. Thus, the coitus-induced sexual-response cycle usually produces a more significant elevation from the base-line pH measurement by the time the resolution-phase observations are terminated.

The castrated subjects have been presented only as a reminder that they represent problems of tremendous individuality and real interest. The surgical castrate lubricates with moderate effectiveness even with a manipulation-derived orgasmic cycle, but is much more effectively lubricated subsequent to coital stimulation. For this individual, the pH of the vagina usually ranges within the previously accepted normal base-line levels (4.0 to 4.9) if adequate estrogen and/or progesterone are ingested. This research subject has provided further support to the thesis of direct humoral influence upon vaginal pH that has been presented over the last 20 years by the original investigators in this field.^{10, 22, 23, 25, 27}

The artificial vagina also presents problems of real, but restricted, interest to the gynecologic discipline. The base-line pH levels of the artificial vagina as previously described by Peterson²¹ rise to the high side of the vaginal pH range (6.5 to 7.5). The present subject has not been observed under controlled conditions for a length of time sufficient to support more definitive statements. The details of her vaginal physiology will be considered, together with those of the surgically castrated subject, in a separate publication.

Summary

If brief, we have an unsolved problem. The anatomical picture associated with the production of vaginal lubrication is established. The true source of this lubricating medium is still in doubt. The effect of vaginal lubrication,

developed with the usual sexual response cycle, upon the pH of the vagina varies with the degree of acidity of the original base-line measurement, the type of sexual stimulation employed, the amount of lubrication produced and the length of productivity, that is, the length of the individual sexual stimulation period. There is real doubt, at present, as to the true pH base line of the vagina, despite multiple previous observations all of which were uncontrolled for possible vaginal contamination.

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THE PERMEABILITY OF THE VAGINAL MUCOSA

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The pioneer student of absorption of chemical substances from the vagina was David I. Macht. While at the Johns Hopkins Medical School, Baltimore, Md., in 1918 he published his classic paper *The Absorption of Drugs and Poisons Through the Vagina*. The following quotations are from that paper.

"The vagina is generally regarded as an organ incapable of absorbing pharmacological agents. Witness to this widespread belief is the universal and indiscriminate employment by women of all kinds of medicaments . . . some known to be innocuous and others recognized, otherwise, as violent poisons . . . in the form of douches, tampons, suppositories, 'uterine wafers,' etc. Such a practice is not only condoned by the majority of medical men but is often deliberately recommended by them, the prevalent opinion among the profession being that the drugs applied to the vagina exert only a local effect and are not absorbed into the system.

"... The question of chemical substances being absorbed through the vagina has up to the present not been definitely settled. No experimental work on lower animals has, as far as the author knows, been done on this subject. The only experimental work on record comes from two clinicians, Hamburger (1876) and Menges (1906)."

The medical profession today entertains a quite different view of the matter, being instead ultraconservative.

Several years ago we at the Ortho Research Foundation became interested in the subject from the standpoint of the formulation and the use of chemical contraceptives (Millman *et al.*, 1950). We were concerned with two matters: (1) spermicides contained in contraceptive gels and creams. These are now known to be absorbed into the system. (2) We were soon convinced that contraceptive gels and creams should not be of such consistency as to allow the spermicide to diffuse out and become absorbed before the moment when they are supposed to perform their protective function.

Whereas experimental studies on vaginal absorption were lacking 35 years ago, medical literature contained many reports of deaths from vaginal douches, suppositories, or uterine wafers containing highly toxic substances. Of the 40 references cited by Macht, 35 relate to such reports. The toxic chemicals include arsenic, mercuric chloride, phenol, iodoform, and belladonna, among others. One item in the literature of special melodramatic interest relates to an Italian who "administered arsenic to his wife through the vagina in order to get rid of her; and, having married a second time, repeated the medication when he became dissatisfied with the second."

Macht performed his experiments on cats and dogs. He felt that dogs are especially favorable experimental animals for such studies because their vaginal mucosa is "histologically practically the same as that of the human being and furthermore because absorption through the uterus can easily be avoided." He included a large variety of substances, noting both the toxicological and

the pharmacodynamic effects and symptoms as proof positive of absorption from the vagina and passage into the blood stream.

It is interesting to note that Baker, the discoverer of phenylmercuric acetate, also used the dog in tests for vaginal irritation of contraceptives; in my view, however, the dog vagina is too resistant for reliable testing.

As further proof of the absorptive capacity of the vagina, Robinson (1927), incidental to his somewhat extensive experimental studies on vaginal absorption, discussed a case of "haematocolpos," or the accumulation of products of menstrual bleeding and tissue breakdown. He calculated that in one case of imperforate hymen of six-years' standing a girl absorbed 200 to 250 ounces, mainly fluid, leaving much solid material behind.

Of more than passing interest among Robinson's observations are the following:

(1) KI was absorbed and recovered in the urine by a woman whose uterus and ovaries had been removed.

(2) After 10 gm. of glucose were inserted into the vagina of a patient, Robinson found no increase of blood sugar and no glucose in the urine; when the same amount of sucrose was administered, some of this disaccharide was recovered in the urine.

(3) Methylene blue and phenol red were found to be absorbed and demonstrable in the urine.

The fact of absorption of substances from the vagina was, therefore, amply demonstrated to the author's satisfaction.

In the meantime, to these lists there have been added various substances of medical significance that have been tested for their transmission from the vaginal cavity into the blood stream.

Thyreotropin. This hypophyseal hormone was found by Aron and Aron-Brunetière (1953) to pass the mucosal barrier, enter the blood stream, and stimulate the thyroid of the guinea pig, a species well adapted for thyroid studies. The effect was checked by histological examination of the thyroid gland.

Penicillin. A number of studies on human patients may be cited, which show not only that this antibiotic is absorbed but that a therapeutic blood level may be maintained as long as desired.

Rock *et al.* (1947) administered 100,000 units of penicillin in cocoa butter suppositories with these results.

(1) In 5 nonpregnant women suffering from vaginitis, the blood level was 0.687 U./ml. in $\frac{1}{2}$ hour; in 9 other women, 0.655 U./ml. in 1 hour.

(2) Very little absorption occurred in 4 women near term.

(3) In 7 postpartum patients 200,000 U. *per vaginam* resulted in a blood level of 0.938 U./ml. in $\frac{1}{2}$ hour.

Therapeutic levels usually were reached in 4 to 6 hours. Rock *et al.* place these levels at 0.039 for streptococci and at 0.078 to 0.100 for staphylococci. For absorption of sulfa drugs see Carrington *et al.*, 1946.

Absorption, it was noted, proceeded more slowly around the middle of the menstrual cycle. It is at this time of the cycle that the corneum layer is the thickest (Davis and Hartman, 1935); during pregnancy in women the corneum

of the vaginal wall is especially thickened, which would seem to explain the slower absorption at these times.

Goldberger *et al.* (1947) and Schudmak and Hesseltine (1951) also met with favorable results in using the vaginal route for administering penicillin.

Quinine. Robinson described the experience of a patient who was extremely sensitive to quinine: even extremely small doses produced marked erythema. On two occasions the patient used a vaginal suppository containing 5 grains of quinine HCl as a contraceptive. On each occasion the insertion of the suppository was followed shortly by a marked erythematous outbreak. The author states that the patient was ignorant of the fact that the suppository contained quinine, and "other cases of a similar nature are on record."

Another proof of vaginal absorption deriving logically from an allergic phenomenon, reported by James (1945), brings me very close to the subject of this paper. This case concerns a pregnant woman suffering from pernicious vomiting on the third day after coitus and being entirely free from nausea at other times. The hyperemesis did not recur when the husband resorted to coitus interruptus.

Proteins. Ingenious experiments by Rosenzweig and Walzer (1943) demonstrated the capacity of the vaginal mucosa to transfer protein material into the blood stream. In a patient allergic to peanut protein, a bit of his or her blood serum was used to cause a passive sensitization of a small spot on the forearm of the subject by intracutaneous injection of 0.05 ml. of a $\frac{1}{10}$ per cent solution of the serum; this constituted the "reagents" for peanut protein.

To perform the test, 5 gm. of raw ground peanuts in 15 ml. of water were taken up in a cotton pledget and placed in the vagina or on the cervix. The sensitized spot on the forearm of the subject was watched for a positive reaction.

Absorption from the vagina was slower than from the cervix, requiring 41 to 120 min. from the former, only 8 to 25 min. from the latter.

The Unidirectional Permeability of the Vaginal Mucosa

Thus far I have cited ample proof that the vaginal mucosa will allow a variety of substances to pass into the blood stream. In this regard little has been added basically since the early experiments of Macht.

Several years ago I approached the problem of vaginal permeability from a new standpoint, advancing the theory that the permeability is essentially unidirectional; the reverse—that is, from blood to vaginal lumen—obtaining only in the case of water. That water passes from the blood and lymph into the vaginal cavity is attested by the fact that women who have had uterus and cervix removed (drainage from above being thus eliminated) still have a moist vaginal wall (Gow, 1894; Robinson, 1927; and others). Parenthetically, it may be noted that the human placenta also offers no barrier action to the passage of water between mother and fetus (Gellhorn and Flexner, 1942) as molecules of water pass readily to and fro across the epithelium of the villi.

The reasoning on which the above-mentioned theory was based arose from my frequent failure to find Cl^- ions in the vaginal fluid; usually, I also found glucose absent in the vagina of the rabbit. In women, glucose enters the vagina with the cervical mucus, most abundantly around the time of ovula-

tion. The presence of glucose in the watery cervical mucus at the time of ovulation has been the basis of the so-called Tes-Tape test for ovulation (Birnberg *et al.*, 1958; E. Tyler, elsewhere in this monograph). This test, in its present form, is not applicable to vaginal use, possibly because the glucose of the cervical mucus with its content of glucose is absorbed too readily with resultant inconsistencies. Practically nothing is known about this.

Reasoning further, it would, of course, be disastrous if glucose and NaCl were lost continuously by this route. It is apparent that for these physiologically essential blood constituents the vaginal mucosa is selective. The vaginal mucosa shares this selective action with many cellular membranes of the body: gland cells and kidney tubules, for example. Most cell membranes, indeed, allow K^+ ions to pass in and out readily, and resist the entrance of Na^+ ions.

The following report on experiments to test the above theory is to be considered preliminary in nature. My studies had to be discontinued at their most interesting stage. A few experiments will be described, which, it is hoped, will serve as a starting point for an extensive project in this area of reproductive physiology.

Several methods were designed to test the reverse passage of substances, from the blood stream into the vaginal lumen: (1) by building up a high level of a given substance in the blood stream and testing for its presence in the vaginal lumen; (2) by injecting drugs or dyes with a hypodermic into the connective tissue spaces about the vagina (Wislocki having demonstrated that in this manner the rich lymphatic supply could also be injected); and (3) by using the excised vagina as a dialysis sac.

I. SYSTEMIC ADMINISTRATION OF TEST SUBSTANCES

KI. In a cat anesthetized with Dial urethane, 2 ml. of a 2 per cent solution of KI was injected intraperitoneally; in another, intravenously. No iodine was recovered from the vaginal cavity, but it was demonstrated in the saliva. The starch test was used.

Glucose. A rabbit was anesthetized with Dial urethane. The Schaffer-Hartmann method was used to detect the glucose. Glucose was administered by the intravenous drip method until a high level of blood sugar was built up. In two experiments no glucose was demonstrable in washings from the vagina at any time.

It was planned to make extensive observations in this area in alloxan-diabetic animals, but circumstances prevented further pursuit of the subject at this point.

Sulfanilamide. A rabbit was anesthetized with Dial urethane and the cervix tied off. The drug was administered by stomach tube until the blood level had reached 17.5 mg. per cent. At no time was the drug demonstrable in the vagina. (For absorption of this therapeutic agent from the vagina, see Carington *et al.*, 1946.)

Neutral red. For an hour after intravenous injection of 5 ml. of a 1 per cent solution of this dye into a rabbit, the vaginal lumen remained free of this material.

Light green. A 1 per cent solution of light green was injected I.V. into a rabbit until the mucous membrane of the mouth, the tongue, and the vulva showed presence of dye (shimmering through). In each of three experiments the vaginal lumen remained free of the dye. Light green, on the other hand, was found to be readily absorbed into the blood stream.

Safranin and methylene blue. The same negative results applied to I.V. injections of 5 ml. of safranin and of methylene blue. The former dose was sufficient to kill the experimental animal.

Antigen and antibodies. Perhaps the only really significant experiment in this area was with the use of human blood serum as antigen and its antibodies. The experiments were performed as follows.

Two weeks after 2 ml. of human serum was injected I.V. into rabbits, the presence of antibodies against human serum was demonstrated in the rabbit blood, but none in the vaginal lumen. However, 2 weeks after the human serum was instilled into the vagina of a rabbit, the animal's blood contained the antibody.

Sulfacridine. A lethal dose (4.37 mg./kg.) of this chemotherapeutic compound was injected slowly into a cat over a period of 30 min. After death of the animal the inner surface of the vaginal wall, viewed with ultraviolet light, was found to be free of the dye.

The few experiments thus far detailed give ample proof that not only simple compounds such as NaCl, KI, and K_4FeO_6 but also large molecules such as serum albumins and other proteins pass readily through the mucous membrane of the vagina. The experiments also indicate that these substances, so far as can presently be proved, do not pass in the reverse direction.

Perivaginal Injections

It was reasoned that if substances were injected in ample concentration into the connective tissue spaces about the vagina and thus brought into direct contact with the "outside" of the membrane, then, if at all, they should move through the mucous membrane into the lumen of the organ.

Sulfacridine. As already stated, the presence of this dye is readily discovered under ultraviolet light. One cat received 5 ml. and a second cat 10 ml. of a 0.6 per cent solution, perivaginally, under Dial urethane anesthesia. Upon dissection of the vagina, the connective tissue side of the organ was seen to be well impregnated by the dye, but none was found on the inner surface.

KI. Of 2.5 ml. of a 2 per cent solution of KI injected perivaginally, none found its way into the vaginal lumen.

Olive oil. A cat was anesthetized with Dial urethane. Fats are known to pass out of the vaginal cavity through the vaginal wall. When as much as 5 ml. of olive oil was injected into the circumvaginal tissue spaces, no fat could be demonstrated within the lumen.

Experiments with the Excised Vagina as a Dialysis Sac

Some experiments were made *in vitro* with the excised vagina set up as a dialysis sac. The solution containing the substance to be tested was placed inside the sac, water on the outside; or, contrariwise, water was placed inside,

the solution to be tested on the outside. Tests were made some hours after setting up the apparatus, the last within 20 hours.

NaCl. The salt passed readily from the inside to the outside. Late in the experiment a small amount of Cl^- ions could be demonstrated as having passed in the reverse direction. This might have been due to the dying condition of the membrane. Cl^- was determined by the Volhard-Arnold method.

Rivanol (an acridine). This therapeutic agent is recognized readily in ultraviolet light.

The vaginal membrane displayed high resistance toward this compound; the drug passed in both directions in very small amounts. Neither direction was favored over the other. It is quite possible that such unphysiological substances as somewhat toxic drugs such as the acridines denature the cells of the vaginal membrane sufficiently to destroy their selective capacity.

This subject deserves further experimentation.

Summary of Section I

Macht's findings of thirty years ago on the ready absorption of a variety of chemical substances from the vagina are confirmed. To the list I have added human serum as an antigen.

In this paper I have introduced as a new concept, the unidirectional transmission of substances from the vagina into the blood stream, with no such transmission in the reverse direction. It must be reasoned teleologically that it would be disastrous for NaCl , glucose, hormones, or amino acids, for example, to be lost by this route.

In three lines of experiments I have attempted to test this concept preliminarily:

(1) By producing a high titer of substances (KI , sulfanilamide, glucose, antibodies) in the blood stream. In all cases the vaginal cavity remained free of the experimental substances.

(2) By saturating the perivaginal connective tissue spaces with the same substances. Negative results were also attained in this series of experiments.

(3) By experimenting with the excised vagina of the rabbit as a dialysis sac. The results with this series were less decisive.

These fragmentary experiments are presented in the hope that their presentation may stimulate some one to make the consideration of the vaginal mucosa as an absorptive organ a more extensive and intensive study. Researches in vaginal physiology have been lagging.

Finally, because of the rapid absorption of substances from the vagina it is clear that contraceptive gels and creams soon lose their effectiveness if they give up their spermicides by diffusion too readily.

II. ABSORPTION OF SEMINAL PLASMA AFTER COITUS

In 1947 Green-Armitage wrote that in his clinical experience he had noticed that the uterus and upper third of the vagina of young virginal women were abnormally small at the time of marriage, but that such uteri grew to the accredited (*sic*) normal size within a few months of married life provided that no contraceptive was employed that interfered with the absorption of semen.

If sheath or coitus interruptus was resorted to, such uterine development did not take place (Green-Armitage *et al.*, 1947).

In 1943 Green-Armitage stated that "anything or any method which prevents, retards, or alters the normal degree of physiological absorption of human semen from the vagina carries with it, during the early months or years of marriage, the risk of sterility from failure of uterine development and proper sequence of uterine secretion."

Commendably, Green-Armitage *et al.* (1947) proceeded to test the effect of seminal injections into immature rats and guinea pigs and achieved considerable growth in about half of these animals. Mistakenly, however, they assumed that the stimulation they observed was to be attributed to testosterone in the semen. These results were contrary to the findings in similar experiments of Bacsich *et al.* (1945).

The idea of beneficial influence to the female organism following vaginal absorption of certain constituents of semen is not new. It is doubtless as old as folklore; W. H. Long in his *Sane Sex Life and Sane Sex Living* (1919) makes much of this theory. No less a scientist than Arthur Thomson (1922) agreed to this concept in a talk delivered at the Neo-Malthusian Birth Control Congress in London in July 1922. This and other papers of a similar character, pro and con, were reported in the *Practitioner* in 1923. In this same journal, Fairham sarcastically remarked that doubtless the Chicago packing houses would soon produce a gland extract in tablet form for local administration as a substitute for semen.

Much nonsense* has been written on this subject in the recent decades. However, there have accumulated in the last decade some pertinent data on the pharmacodynamic action of seminal plasma, together with isolation of several of the active principles. These concern the stimulating action of seminal plasma on the smooth musculature of the uterus, with identical action on the gut. It is this oxytocic action that especially concerns us here; but, as Mann has pointed out (page 26 of his book, 1955), the active compounds in semen, arising as they do in the seminal vesicles and the prostate gland, probably play their most significant role in the emptying of the glands in ejaculation, as shown by their decreased ability to contract once more until enough of the freshly formed secretion has accumulated again (Cockrill *et al.*, 1935; Martins and Valle, 1939; Karlson, 1949; Huggins *et al.*, 1945; and Brügger, 1945).

We owe the pioneering biochemical studies on the oxytocic properties of semen to von Euler (1934, 1936, 1939) and to Goldblatt (1935a, 1935b), especially the former, who analyzed the semen of several species of mammals: rat,

* The following additional hard-to-document, even bizarre claims may be cited. Mayer (1950) claims that total semen reaches the Fallopian tube and thence the abdominal cavity, citing as proof increase in ascitic fluid due to peritoneal irritation by sperms. The author overlooks the fact that the amount of fluid in the Douglas' cul-de-sac varies with the cycle. "Young female singers do not attain full development of their voices until they are married."

Stemmer (1940) claims that sperms cause endometritis. In the mare, he says, as in women, hormones are absorbed from semen in the posterior fornix. Answer: in the mare the semen is catapulted directly into the open uterus and little semen is ever found in the vagina after copulation. The author further claims that orgasm stimulates the thyroid and that thyroid hormone synergizes with "hormones" absorbed from semen after coitus. Colmeiro-Laforet (1954) also postulates penetration of whole semen into the uterus and the Fallopian tubes, and notes the odor of semen in the woman's breath after coitus!

guinea pig, rabbit, bull, man, and monkey. I had sent him the monkey semen from my rhesus colony at the Carnegie Laboratory in Baltimore, Md., about 1935.

Von Euler isolated two chemical entities with oxytocic activity: prostaglandin from the prostate and vesiglandin from the seminal vesicles. He determined the chemical structure of the former and found it to be nitrogen-free. My colleague Singher, with Tyler (1954) of Los Angeles, Calif., found that when semen is separated into two portions, one containing protein and one free of protein, both fractions possess oxytocic activity. The conclusion is that probably there is a number of substances in semen exhibiting oxytocic activity.

Asplund (1947*a*, 1947*b*) would add nonspecific compounds present in semen to explain the stimulating effect on uterine muscle of, for example, choline (Mann, page 188) and another substance unaffected by atropine. Also, in view of the reported synergistic relationship between adrenaline and noradrenaline and seminal plasma (Goldblatt, 1935), as tested on the isolated seminal vesicle of the guinea pig, it is tempting, teleologically, to postulate the purposiveness of this synergism in assuming a more effective emptying of the accessory glands due to the stimulation of the adrenal medulla during sexual excitement. One further item of adaptiveness in this area is the demonstrated more certain advance of sperms in the female genital tract upon stimulation of the uterine musculature.

The chemical studies of von Euler were preceded by the demonstration of pressor effects of prostatic secretion and oxytocic action of human semen on strips of human uterus by Kurzrock and Lieb (1931). This subject is discussed in some detail by Mann (pages 26 and 27).

While Green-Armitage was probably mistaken in assigning uterine growth effects to male hormones in seminal plasma, it has since been amply demonstrated that there is present in human semen a modicum of estrogens. These have been characterized by Diczfalusy (1954): a liter of semen would have approximately 60 μ g. of estrone, 10 of estradiol-17 β , and 30 of estriol. In terms of estradiol activity equivalence there thus might be in one human ejaculate the estrogenic activity of 0.1 μ g. of estradiol. If *all* of this were absorbed from each ejaculate twice a week there could be no appreciable speeding up of the normal growth of the uterus.* (See also Dirscherl and Breuer, 1955.)

This is approximately the present status of the physiological effects of absorption of seminal plasma from the vagina after unprotected sexual intercourse. It is clear from this unfinished study that much careful and thorough work must be done to understand the physiology of the vagina.

ADDENDUM

Since compiling the data for this paper I have received a copy of the extensive monograph on the pharmacology of the seminal oxytocics by Rune Eliasson: *Studies on Prostaglandin:*

* Some additional references on hormones in semen: in human semen Riisfeldt (1948) finds some estrogen, Natoli (1950) none; Schaffenburg *et al.* (1954) find estrogen; in a former study, McCullagh and Schaffenburg (1951) demonstrated the presence of both female and male hormone, the latter causing increase in phosphatase in the prostate gland; in bull semen Puntriano (1953) finds testosterone, but no estrogen.

Occurrence, Formation and Biological Actions, in *Acta Physiol. Scand.* Vol. **46**, Suppl. 158, 73 pp., 1959. Among other contributions the author shows that prostaglandin is elaborated by the seminal vesicles, not by the prostate.

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HISTOPATHOLOGY OF SURGICALLY EXCISED LESIONS OF THE VAGINA

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Clinicians agree, generally, that the vaginal tube is a simple, medically uninteresting organ. Because of its relative freedom from major disease, it is regarded by the gynecologist as the site of mild parasitic invasion, as something to be repaired after obstetrical delivery, or as the route by which other genital organs are examined. I shared these feelings of disinterest until recently when a rare lesion, to be described below, was discovered in histological material coming through this laboratory. As instances of vaginal disease were assembled for this study from records at Charity Hospital, New Orleans, La., it was no surprise to see the list grow in number and variety as more unusual diseases were brought together.

The purpose of this report is to illustrate and summarize histological features of lesions of the vagina from material available, and to point out several new examples of unusual mutation of the genital tract epithelium.

Material

All charts for all coded diseases of the vagina from January 1, 1945 to December 31, 1958, were drawn from the medical records index at Charity Hospital. A list was prepared comprising all types of lesions represented in this series of diseases (TABLE 1). From this list and from available references in medical literature, a classification of surgical diseases was assembled (TABLE 2).

Histopathology

It would be desirable, especially from the clinical point of view, to distinguish between nonneoplastic and neoplastic tumors. This difference is best determined histologically. These lesions are considered nonneoplastic:

Primary endometriosis. This usually small nodule may appear grossly as a firm, solid, or a blue-purple cystic mass, tender to palpation, and located in the fornix or in the vaginal vestibule. Microscopic section (FIGURE 1) shows typical endometrial glands and stroma. It is to be expected that during pregnancy responsive endometrial tissue in these nodules would undergo decidual change and thus become more prominent to the examiner. FIGURE 2 illustrates decidual change in an endometrial nodule in the vagina. Another change occurring in ectopic endometrium is illustrated in FIGURES 3 and 4, tubal metaplasia. This type of metaplasia has been observed in the uterine lining. Its cause and meaning are not known, but it is certainly illustrative of the power of Müllerian epithelium to change form.

Mesonephric cyst. The single Gartner's duct cyst is often mistaken for an inclusion cyst by the clinician. The expected type of lining of this cyst is low columnar, but actually there seems to be a variety of epithelia, both columnar and stratified squamous, found in these. It is possible that such an assortment of types has been reported simply because cysts of other kinds have been in-

cluded in this class. However, it is also likely that the original epithelium exhibits a potential for forming other kinds. To have originated in the mesonephric duct, a cyst must be located laterally or posterolaterally in the vagina. FIGURE 5 illustrates the lining of a mesonephric cyst with tall columnar epithelium.

TABLE 1
VAGINAL TUMORS BY TYPE

Gartner's duct cysts	15
Primary squamous cell carcinoma	10
Metastatic squamous cell carcinoma (initial diagnosis)	8
Leiomyoma	5
Metastatic adenocarcinoma	5
Inclusion cysts	3
Fibroma	3
Condyloma (squamous papilloma)	3
Metastatic mixed Müllerian tumor	2
Metastatic transitional cell carcinoma	2
Skene's duct cysts	2
Endometriosis	2
Sarcoma botryoides	1
Adenocarcinoma, paramesonephric	1
Choriocarcinoma	1
Hemangioma	1
Cyst with Müllerian-type epithelium	1
Unclassified tumors	12
"Vaginal cyst"—unspecified	7
Total cases	84

TABLE 2
TUMORS OF THE VAGINA

I. Nonneoplastic tumors	III. Malignant neoplastic tumors
(1) Primary endometriosis	(1) Primary
(2) Solitary cysts	(a) Squamous cell carcinoma
(a) Mesonephric-type cyst	(b) Adenocarcinoma
(b) Paramesonephric-type cyst	(c) Sarcoma
(3) Paramesonephric adenosis	(2) Metastatic
II. Benign neoplastic tumors	(a) Squamous cell carcinoma
(1) Squamous cell papilloma	(b) Adenocarcinoma
(2) Fibroma	(c) Choriocarcinoma
(3) Myoma	
(4) Hemangioma	
(5) Rare neoplasms	

Paramesonephric cysts. The vaginal cyst lined by mucinous columnar epithelium of the kind found in the endocervix is a familiar type. At times the epithelium is identical to that of the endocervix (FIGURE 6). With such strong morphologic identity, it would be reasonable to assume that the tissue of origin is paramesonephric (Müllerian). In our experience mucinous epithelium is seen as the most frequent lining of vaginal cysts. Evidence will be presented indicating that such cysts and, indeed, adenosis of this type may be acquired by extension of endocervical epithelium, and not necessarily by development from tissues misplaced in the genesis of the genital tract.

Paramesonephric adenosis. Von Preuschen¹ appears to have been one of the early observers of this entity, when in 1877 he described typical mucous glands in the vagina. Bonney and Glendining² recorded a description of the condition which they called adenomatosis vaginae. It is noteworthy that the patient

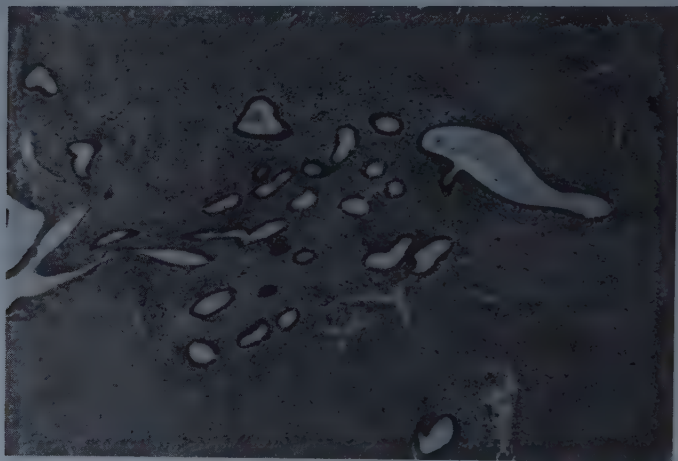


FIGURE 1. Endometriosis of the vagina.

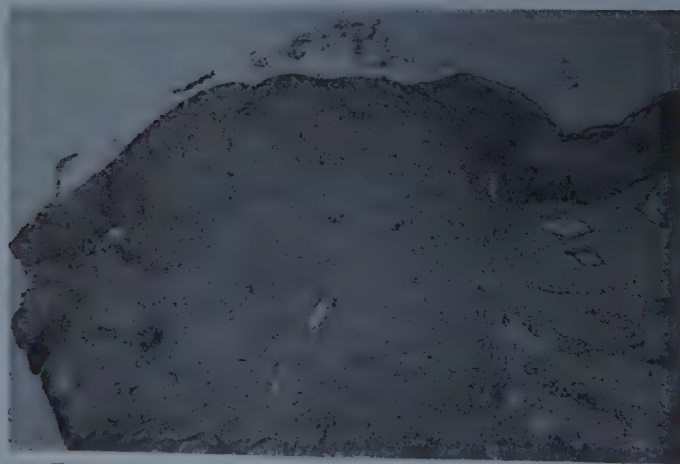


FIGURE 2. Endometriosis of the vagina with decidual change.

consulted Bonney for the same symptom which compelled the following patient to seek help.

Illustrative case history No. 1: B.P., a 21-year-old white female was first seen in March 1953 for excessive mucous vaginal discharge of about 6-month's duration. Operation was performed elsewhere 8 years prior for pelvic mass which proved to be hematometra in the right body of a double uterus. X ray visualized a solitary left kidney. Examination revealed double vaginas with

normally pink, rugous membranes and double cervices. Under observation with speculum in place, mucous secretion was seen to well up in the fornix on both sides of the septum. Biopsy of the right vaginal wall in 4 places between fornix and vestibule disclosed epithelial changes identical with those seen in



FIGURE 3. Endometriosis of the vagina with tubal metaplasia.



FIGURE 4. Tubal-type epithelium and papillary folds.

erosion healing in the cervix (FIGURES 7 through 10). A ragged layer of stratified squamous epithelium covered the surface, while many mucous glands opened onto it.

The abnormality in this instance is almost identical with that described by Bonney. The history of onset in the two patients seems to indicate an acquired disease. The fact that there were other developmental defects in the latter case could mean a congenitally anomalous vaginal mucosa, although this

assumption would be guilt by association. However, it is entirely possible that cervical gland epithelium grew down onto the vagina in the same way that it covers the portio of the cervix in erosion healing. This would provide an easy explanation for the genesis of both solitary cysts and adenosis. Routine histological examination of the upper vagina is now being carried out in this



FIGURE 5. Mesonephric cyst of the vagina lined by tall columnar epithelium.

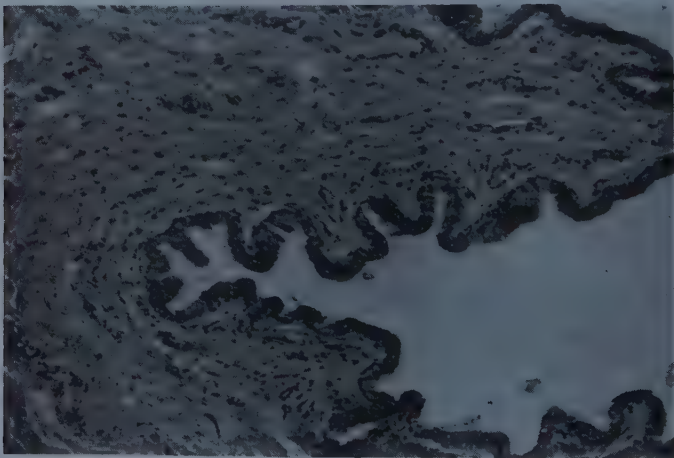


FIGURE 6. Paramesonephric cyst of the vagina lined by mucous-type epithelium.

laboratory in conjunction with a radical surgery program with the aim of discovering symptomless abnormalities of this kind and of determining their true frequency.

Benign Neoplastic Tumors

Benign neoplastic vaginal tumors come under the scrutiny of the histologist when removed either incidental to other gynecological procedures or because

they occupy space needed for other purposes. The specimens submitted in our material were usually small, being removed and sent in by a curious clinician. Some benign tumors claim attention because they resemble malignant growths. The following benign neoplasms should be noted.

Squamous papilloma (squamous cell papilloma). By definition this should be a benign neoplasm made up of proliferated squamous epithelium supported by

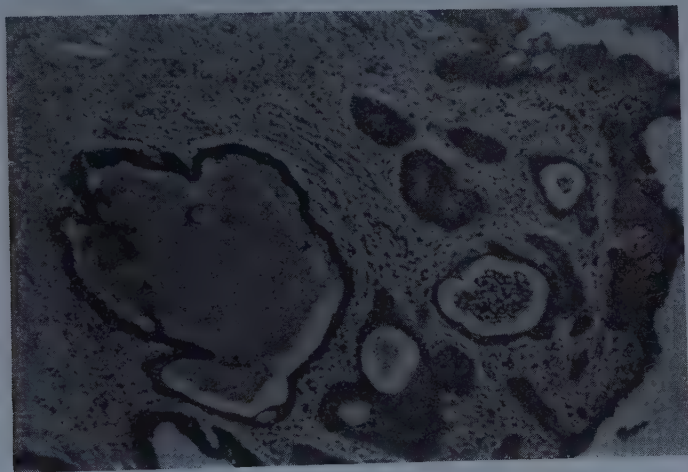


FIGURE 7. Paramesonephric adenosis of vagina.

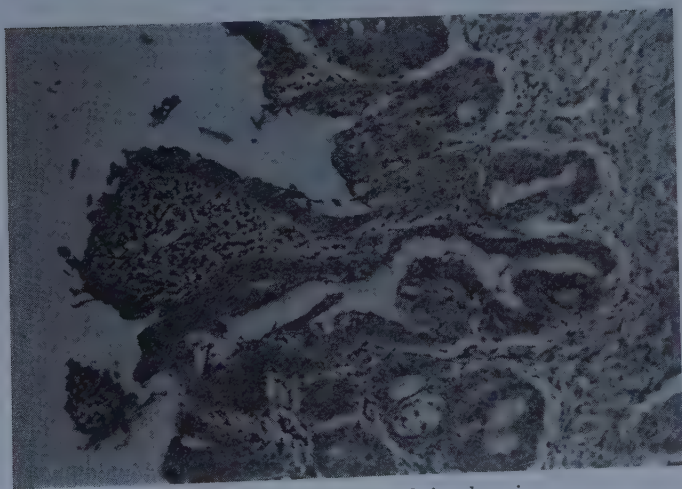


FIGURE 8. Paramesonephric adenosis.

a connective tissue stalk and projecting outward from the surface. There has always been difficulty in distinguishing this entity from condyloma acuminatum, if indeed there is a real difference.³ Condyloma acuminatum is a form of infectious, autoinoculable wart that has a predilection for the genital and perianal regions; hence it is referred to as a venereal wart.⁴ It occurs, among

other places, in the vagina and vaginal vestibule; it is usually multiple, rapidly developing, and amenable to chemotherapy. A virus is said to be the etiological agent. The squamous papilloma, on the other hand, is often single, appears in older women, and is refractory to local medicinal treatment. The

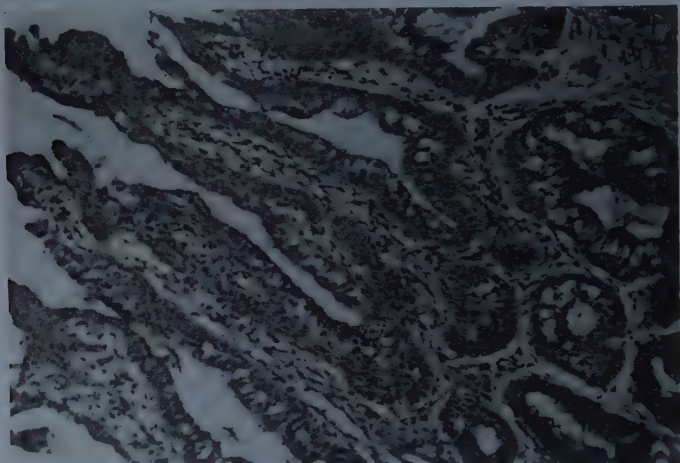


FIGURE 9. Mucous-type glands and squamous metaplasia in the vaginal wall.

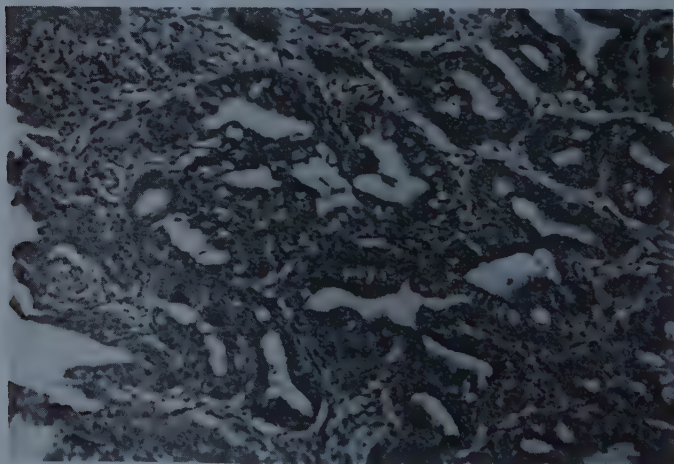


FIGURE 10. Another area of mucous-type glands, paramesonephric adenosis.

squamous cell papilloma seems to have a slight cancerogenic potential, although this has never been documented. It will be noted that all these characteristics are clinical.

Histologically, there seems to be little difference between the two. Both growths consist of hyperplastic squamous epithelium arranged in rolling festoons on a thin vascular connective tissue stalk. There may be pseudo-

epitheliomatous hyperplasia or atypical cellular hyperplasia of the epithelium. When these findings are present there is real danger of confusing the lesion with carcinoma. Furthermore, the opposite mistake of interpreting a papillary squamous cell carcinoma as a squamous papilloma may be made.

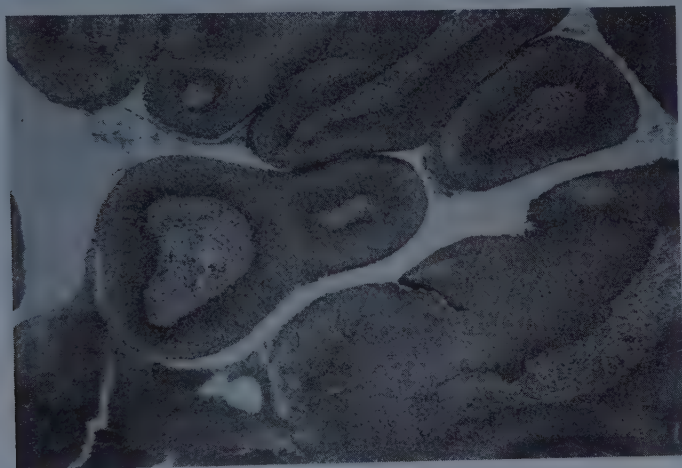


FIGURE 11. Papillary lesion of vaginal wall, Case No. 2.

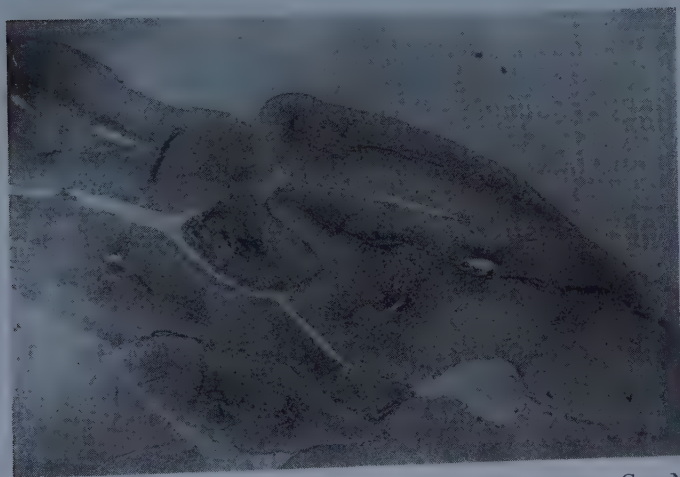


FIGURE 12. Same lesion as in FIGURE 11, removed by hysterectomy, Case No. 2.

Illustrative case history No. 2: L.S., age 63 years, was seen for vaginal bleeding. A verrucous lesion found on the cervix and upper vagina was biopsied on December 22, 1949, under anesthesia. The histological diagnosis read "squamous papilloma" (FIGURE 11). Abdominal hysterectomy was done with removal of the upper vagina on January 10, 1950. The pathologist was of the opinion that the growth was "squamous cell papilloma, consistent with condyloma acuminatum" (FIGURE 12). The patient returned three years later

with a fungating mass in the upper vagina and infiltration laterally to both pelvic walls. Biopsied, this mass was interpreted as squamous cell carcinoma (FIGURE 13), but the pattern was the same as that of the earlier specimens. Full radiation treatment produced five-year survival in this patient.

Fibroma. All 3 of the fibromas in this series were small, less than 2 cm. in diameter. The gross and histological appearance is the same as that of fibromas growing elsewhere. Connective tissue fibers and cells grow compactly and in a circumscribed manner. Degeneration of the collagen is common. One tumor was associated with ovarian thecoma.

Myoma. This neoplasm occurs more commonly than the preceding and possibly grows more actively. Appearing in the latter part of the reproductive age, the myomas observed at Charity Hospital were 3 to 6 cm. in diameter. Smooth-muscle differential staining proves the nature of the tumor.



FIGURE 13. Squamous cell carcinoma, Case No. 2.

Hemangioma. Several of the tumors sent through the laboratory were of unusual structure and, although benign, defied classification. One of these was signed out as hemangioma with marked sclerosis. It was composed of spindle cells, resembling fibroblasts, arranged in fascicles and bundles spaced apart by hyalinized material devoid of structure (FIGURE 14). There were numerous small- and medium-sized blood vessels, mostly lined by a single endothelial layer.

Typical capillary angiomas also occurred in the vaginal wall.

Malignant Neoplasms

Malignant neoplasms are most often metastatic. The vagina is, indeed, a common site for the spread of uterine neoplasms. Of far greater histological interest are those tumors primary to the vagina. The following malignancies occur.

Primary squamous cell carcinoma. Ten primary squamous cell carcinomas

were found among our patients. Although not as common as metastatic malignancy, this type of growth is the usual primary malignancy of the vagina. The tumor appears as papillary exophytic, nodular indurated, or as an ulcerated lesion. Microscopically, all of these appeared less differentiated than the corresponding neoplasm of the vulva or skin. There are no histological features that differentiate vaginal carcinoma from cervical carcinoma. The site of origin is determined by gross examination.

Primary adenocarcinoma. Having already noted the variety of gland structures in the vaginal wall, one may predict the finding of adenocarcinoma. Meyer⁵ is credited with the first report of a carcinoma of the ampulla of the mesonephric duct. Additional observations have been recorded in both foreign and American writings (Huffman,⁶ Novak *et al.*,⁷ and Studdiford⁸). Studdiford described a type of adenocarcinoma for each of the two known gland forms,

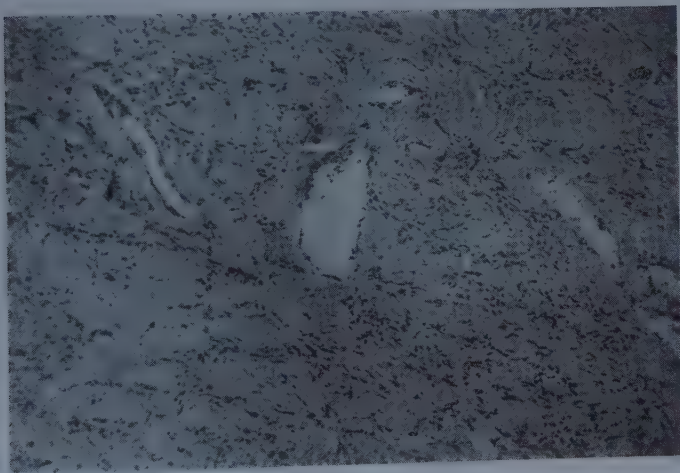


FIGURE 14. "Sclerosing hemangioma."

while the others attributed the origin of tumors in their cases to mesonephric epithelium. One can foretell immediately the difficulty in proving definite vaginal origin of such adenocarcinomas. Unless the growth can be examined thoroughly in an early stage, it may be impossible to assign a definite point of origin. As vaginal adenocarcinomas are rarer than cervical growths, the assertion of vaginal origin would require proof. There was one adenocarcinoma assigned as primary vaginal in our material. Its origin was paramesonephric (FIGURE 15).

Histological structure of vaginal adenocarcinomas as recorded by the above authors suggests the tissue from which the neoplasm begins. In those of mesonephric genesis, the pattern is small glands and tubules closely placed and composed of low columnar, small, darkly stained epithelial cells. While predominantly single-layered, the gland spaces may show piled-up or tufted proliferations of cells. It is noted that this growth is not similar to the usual malignancies arising from mucous glands (endocervical, some endometrial).⁶

Novak remarks on the resemblance to the Schiller mesonephroma.⁷ An adenocarcinoma of this variety may be recognized by its histological make-up.

The microscopic appearance of the paramesonephric or Müllerian adenocarcinoma growing in the vagina presumably would be the same as that of one growing in the endocervix, or possibly similar to endometrial adenocarcinoma, both of these being neoplasms of Müllerian epithelium. Studdiford's case is described as "a well-differentiated papillary adenocarcinoma suggesting the type arising in the cervix."⁸

It is only prudent to note that the classification of a neoplasm on the basis of its cellular and architectural morphology is not infallible. The epithelium of the paramesonephric tract pre-eminently is capable of producing a variety of cells, as noted in squamous metaplasia, in endometrial hyperplasia, and the polymorphism of uterine and ovarian tumors. A neoplasm that looks like one

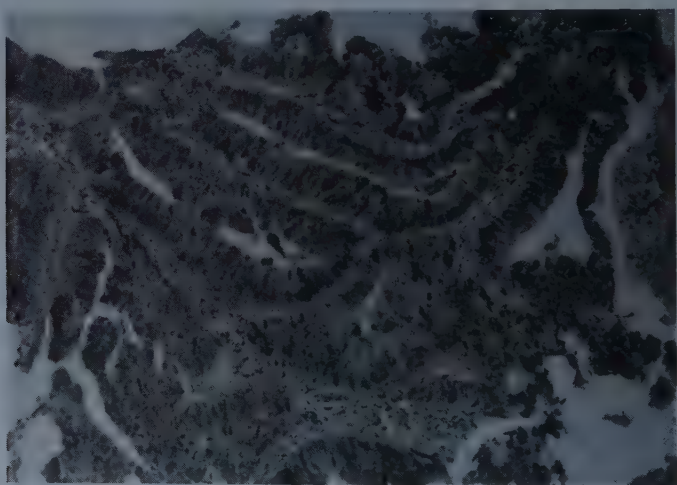


FIGURE 15. Primary adenocarcinoma of paramesonephric origin.

type of normal tissue may have its origin not in that tissue, but in another related type. It must be admitted, therefore, that primary adenocarcinoma of the vagina arising in paramesonephric epithelium cannot be identified with certainty by its histological appearance alone.

Primary sarcoma. There are undoubtedly rare instances of connective tissue and smooth-muscle sarcomas of the vagina, inasmuch as these tissues are present in the wall. However, mention of sarcoma of the vagina brings to mind the more interesting sarcoma botryoides.

Illustrative case No. 3:* A.F.C., a 5-year-old Negro child was seen for vaginal bleeding of two-month's duration and foul vaginal discharge. The examining doctor noted purplish-red tissue masses extruding from the vagina. A lower abdominal mass was palpable and estimated to be 8 cm. across. With the aid of anesthesia he determined that the tumor seemed to grow from the cervix

* Previously reported by Sternberg.⁹

and upper vagina. Local excision of the polyps by electrocautery followed by pelvic exenteration comprised treatment.

Grossly, the tumor is composed of polypoid masses clustered in a manner suggestive of a bunch of grapes. Histologically, most of these polypoid masses are seen to be covered with stratified squamous epithelium of extremely hyperplastic form. Clefts are lined by this epithelium, and there is pseudoepitheliomatous invasion of the mass by long tongues of squamous epithelium (FIGURE 16). The tumor cells are loosely arranged, and there is edema of stroma. Some cells are spindle, undifferentiated type, others are strap-shaped, while still others appear to be so-called rhabdomyoblasts. In this particular tumor there is not so much pleomorphism as there is in the uterine version of the same growth. Under high-power magnification many of the strap-shaped cells are found to have cross striations, indicating that a portion of the growth

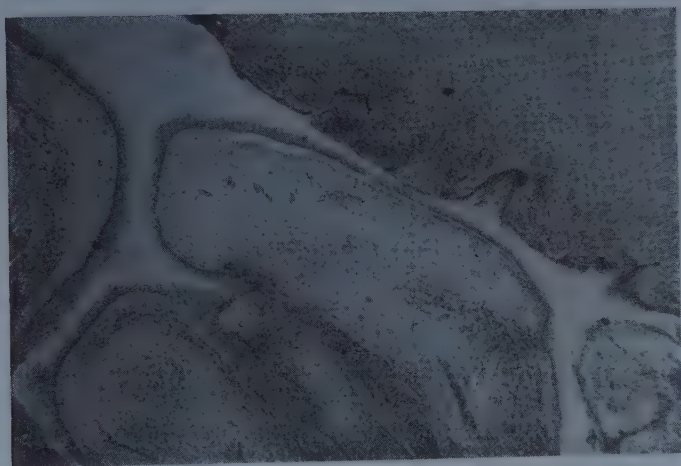


FIGURE 16. Sarcoma botryoides of the vagina.

is rhabdomyosarcoma. Sternberg⁹ has emphasized the frequency of this component of the mixed mesodermal sarcomas.

Metastatic squamous cell carcinoma. Most frequent among the malignancies of the vagina, this tumor spreads from its origin in the cervix or the vulva. There is greater interest in the spread of preinvasive carcinoma to the vagina now that much attention is directed to carcinoma *in situ* of the cervix. A normal-appearing vaginal fornix may be demonstrated, by Schiller stain, to be crusted with surface carcinoma. In this case, the biopsy will prove surface growth similar to the primary on the cervix (FIGURE 17).

Invasive squamous cell carcinoma can be seen in biopsy of areas affected by direct extension of a growth primary in adjacent structures. The histological picture is the same in these areas as in the original organ.

Metastatic adenocarcinoma. Endometrial adenocarcinoma extends to the vagina by lymphatics and by direct growth. Although metastasizing late in its course, this tumor often involves the vagina. Slow growth characterizes metastases as well as the primary growth.

Illustrative case history No. 5: M.D., 75 years of age, complained of vaginal bleeding. She had had previous episodes of off-and-on bleeding for the past 30 years. She had a bad heart, and was refused operation because of this affliction. One year ago she was treated elsewhere with a large dose of intra-



FIGURE 17. Carcinoma *in situ* of vagina extended from cervix.

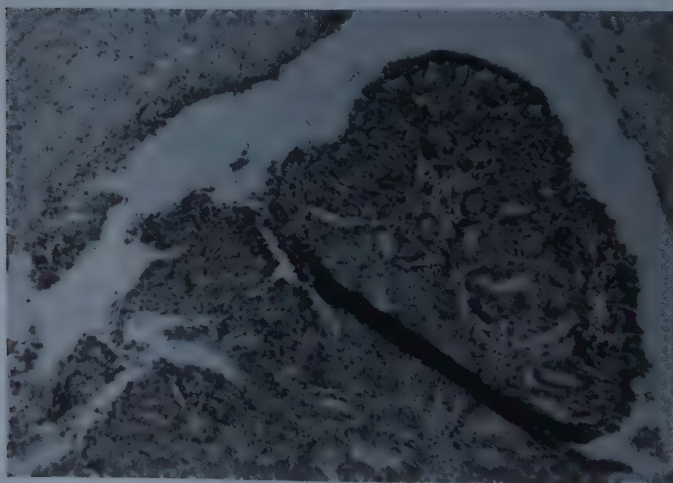


FIGURE 18. Metastatic adenocarcinoma of endometrium.

uterine radium after curettement demonstrated adenocarcinoma. No metastases were evident. The present bleeding, the first since treatment, began 2 weeks before examination. A raised papillomatous lesion was discovered on vaginal examination just within the vestibule. Within the wall beneath the nodule there was an area of induration 2 cm. across. The cervix was small; the uterus, not clearly outlined but not greatly enlarged. X rays showed

multiple lung metastases. Excision of the vaginal nodule verified that it was adenocarcinoma (FIGURE 18).

Rectal adenocarcinoma may produce, as its first symptom, a mass in the rectovaginal septum. There may be a rectovaginal fistula formed as an early manifestation of this disease. Biopsy of the edges of such a fistula or excision

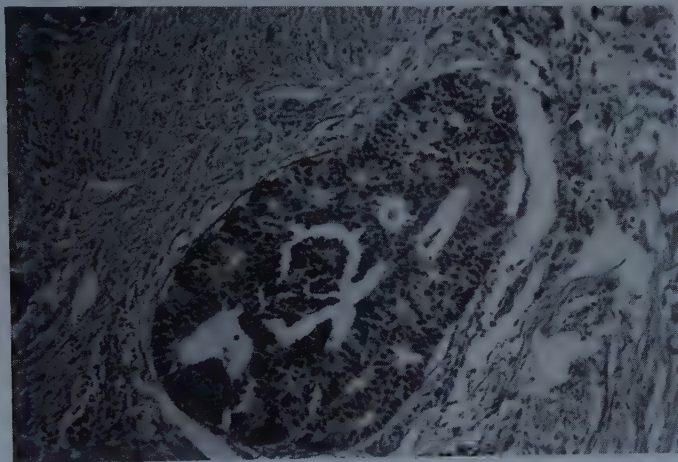


FIGURE 19. Metastatic adenocarcinoma of rectum.

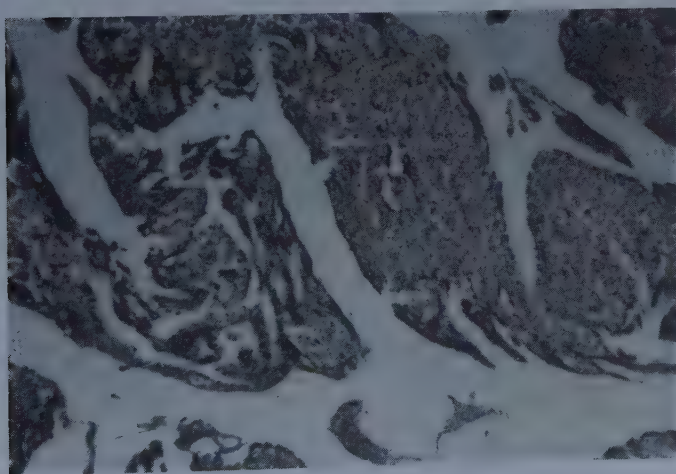


FIGURE 20. Metastatic choriocarcinoma.

of a vaginal wall nodule may be the first proof of adenocarcinoma of the rectum. More practically speaking, proof of spread to the vaginal wall will govern the extent of surgical operation to remove tissue beyond the limits of neoplastic growth. FIGURE 19 illustrates rectal mucinous adenocarcinoma invading vaginal lymphatics.

Metastatic choriocarcinoma. A red-purple nodule in the vaginal wall may be an early sign of uterine choriocarcinoma.

Illustrative case history No. 6: M.W., a 14-year-old Negro, was admitted for febrile illness. On routine examination the examiner noted red fleshy nodules around the urethra. Review of history brought out that the patient had sexual intercourse a year previously and some time later had irregular bleeding. Biopsy of the vaginal lesion proved the diagnosis of metastatic choriocarcinoma. Total hysterectomy and radiation constituted treatment. The patient died of the malignancy 6 months after admission.

The histological appearance of choriocarcinoma is as distinctive in the secondary as in the primary location. Sheets of cytotrophoblast, as well as masses of syncytium, make up the tumor (FIGURE 20).

Summary

This tabulation of histopathology of vaginal tumors is proof that the organ is far from being uninteresting pathologically. It would be difficult to assemble a more varied group of diseases of a comparably simple organ of the body. The fact that there are many normals for each abnormality should not dull the acuity of the clinician in examining the vagina since, after all, the clinical examination is the source of all histopathological material.

Acknowledgments

I thank Nadalyn Cotten for her assistance in summarizing clinical records and preparing special histological sections used in this study. Routine histological sections were kindly loaned by the Department of Pathology, Charity Hospital.

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EFFECTS OF ANTIGENS ON THE BASAL CELLS OF THE VAGINAL SMEARS

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The basal cell of the vaginal smear may have a different appearance under certain circumstances. The cytoplasm may be more dense, more basophilic, and may contain fine vacuoles. This phenomenon was first observed in patients with cancer of the uterine cervix prior to treatment. When these cells were numerous, the patient usually responded favorably to radiotherapy; when they were few, the salvage rate was poor. Because the presence of these cells seemed to indicate that the patient and the tumor were particularly vulnerable to the radiation, or were sensitized, they were referred to as sensitization-response (SR) cells.

A differential count of 100 consecutive epithelial cells in the vaginal smear will indicate the frequency of SR cells. In patients the critical level is found between 9 and 10 per cent. Those below that level are said to have poor SR, those above, marked SR. In TABLE 1 are indicated the 5-year cure rates in patients with cervical cancer, divided according to SR. The over-all cure rate of those treated radiologically is 18 per cent in poor SR and 69 per cent in marked SR. A reverse trend is seen in patients treated surgically: 69 per cent of those with poor SR and only 43 per cent of those with marked SR lived 5 years. The SR correlates with lymph node metastases in Stages I and II, for only 16 per cent (18 of 113) with poor SR have positive nodes, while 61 per cent (20 of 33) with marked SR have positive nodes.¹

SR cells are seen occasionally in women without cancer, but rarely in significant numbers. In a group of 45 women with high SR and no cancer, 25 had an inflammatory lesion (such as cervicitis and vaginitis); 10 were receiving hormones; 6 had allergies; 2, cirrhosis; 1, diabetes; and 1, severe transfusion reactions.

SR cells are elicited in animals with regularity by the administration of antigens. The antigens may be instilled in the vagina or injected intravenously (FIGURE 2). Mouse 2A was observed for 12 days with 9 vaginal smears; none had SR cells. Diphtheria toxoid, 30 Lf in 0.2 ml., was instilled daily in the vagina for 4 consecutive days and, after a 3-day interval, instilled once again. No response was noted until 4 days after the last instillation, when 29 per cent of the cells showed SR. This fell off to 0, rose again to 14 per cent and, following another instillation on day 35, rose again, beginning on day 38, to a maximum of 37 per cent 3 days later. The SR then gradually subsided to zero. On day 74, another instillation was followed by a rise, first noted on day 76, to 44 per cent maximum on day 80. The time relationships between instillation and the appearance of SR cells in the smears is reminiscent of an immune response. No reaction was noted until 10 days after the first instillation. Subsequent responses were noted within 2 to 3 days. In addition, a single instillation sufficed to bring out the response after the initial sensitizing series. Five mice, observed from 90 to 130 days, showed a similar response.

Similar reactions have been observed in rabbits and guinea pigs. An attempt to repeat the experiment in 3 young women was unsuccessful.

Systemic administration of an antigen will produce SR in the vaginal smear (FIGURE 3). A rabbit was given diphtheria toxoid, 40 Lf in 1 cc., intravenously on 5 successive days after a control period of 20 days. Three sera were nega-

TABLE 1
PROGNOSIS IN PRIMARY CANCER OF THE CERVIX

	Poor SR		Marked SR	
	No.	5-Year cure	No.	5-Year cure
Radiotherapy Stages I to IV	93	17 (18%)	54	37 (69%)
Stages I and IIa	34	9 (27%)	25	19 (76%)
Radical hysterectomy and regional lymphadenec- tomy				
Stages I and IIa	99	68 (69%)	30	13 (43%)

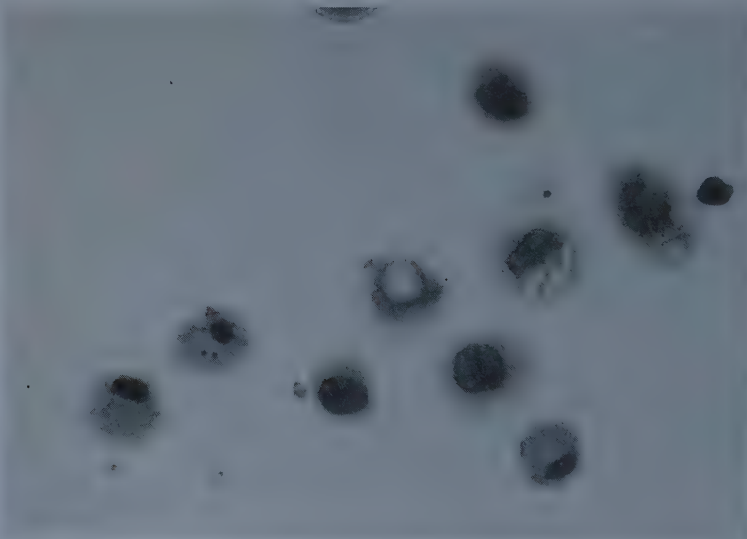


FIGURE 1. Field from rabbit vaginal smear after immunization with diphtheria toxoid. The vacuolated basal cells show the SR. The two degenerate basal cells with pyknotic nuclei at lower left are the usual type of basal cell encountered in rabbits.

tive for antibody, and 11 smears showed SR twice, one at 2 per cent and the other at 4 per cent during the control period. Fifteen days after the first antigen was given, an antibody titer of 1/256 was found; this rose gradually to a level of 1/1024, and then subsided. An SR response up to 10 per cent was noted 24 days after the first antigen; this also subsequently regressed. On days 56 and 57 of observation, 2 more intravenous injections of antigen were

made. The SR rose to 50 per cent on day 58. The circulating antibody titer rose on day 59 to 1/2048, and 3 days later was 1/8192 or more, where it remained for 10 days and then subsided moderately. A single vaginal instillation of antigen on day 83 produced no effect on the serum antibody or SR. However, both were elevated at the time. Similar results were obtained with 2 other rabbits given diphtheria toxoid and with 3 given human gamma globulin.

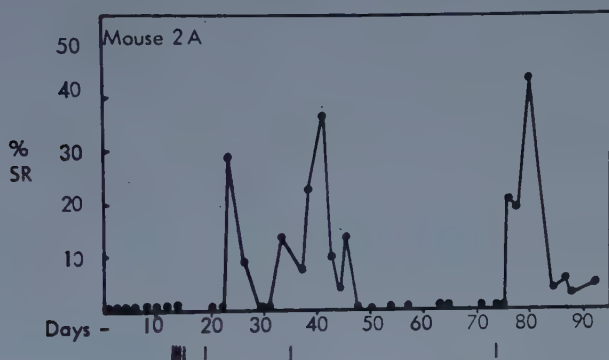


FIGURE 2. Diphtheria toxoid, 30 Lf, 0.2 ml., intravaginally.

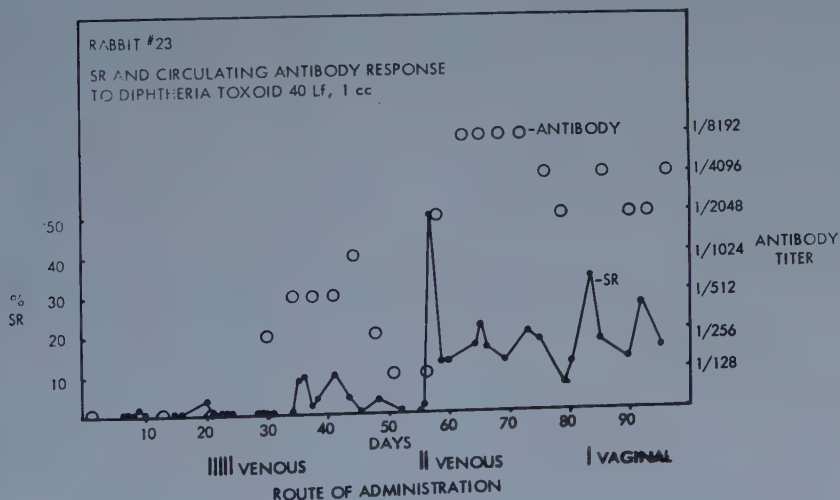


FIGURE 3.

The SR response to globulin was less intense and sustained than to the diphtheria toxoid.

Four mice had an intravaginal instillation of 0.2 ml. of 10 per cent copper sulfate; 2 animals, 0.2 ml. of xylol; and 4 animals, 0.2 cc. of mineral oil and Arlacel A (an emulsifying agent). These agents were administered daily 5 days a week for 2 weeks. A 2-week control period of observation preceded and followed the instillation. The 4 that received the mineral oil and Arlacel A showed no SR. Of the 4 that received copper sulfate, a maximum level of

SR was 5, 3, 12, and 0 per cent in the second week. The 2 receiving xylol showed 3 and 13 per cent SR in the second week. The animals that received copper sulfate or xylol exhibited very bizarre changes in the vaginal smear, but the SR changes were regarded as trifling and not comparable to those obtained with antigens.

These experiments have been selected as representative from a larger number. Substantial series have had the smears counted without knowing when or if antigen was administered. The mice in the first experiments referred to above were part of such a blind series. The regularity of the results leaves no doubt that SR is a consistent observable phenomenon.

The failure of SR to appear following the first administration of an antigen, its appearance about ten days later, and its prompt reappearance after subsequent single applications are similar to immune responses such as that of circulating antibody, and in fact closely parallel the antibody response. The occurrence of modest levels of SR in animals not deliberately given antigen corresponds to modest levels of circulating antibody that may be found in animals prior to stimulation. SR can be elicited by a variety of antigens, and all SR cells look the same.

The sensitization response is not a simple inflammatory reaction in that irritants such as copper sulfate or xylol elicit only a feeble response.

We conclude that the sensitization response is a cellular immune response. It can be elicited by a variety of antigens, by some more effectively than by others. It is not elicited by nonantigenic irritants.

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VAGINAL KERATINIZATION *IN VITRO**

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Introduction

The aim of this investigation is to determine the hormonal and/or nutritional factors responsible for the histological differentiation of epithelial tissues. The rodent vaginal epithelium is of special interest as a target organ for the study of such interrelationships because of its extreme sensitivity, both to vitamin-A deficiency and to estrogen administration. The process of vaginal keratinization produces a similar histological picture, whether caused by a deficiency of vitamin A or by estrogen administration. Vaginal keratinization may represent, however, a common end point for different histogenetic processes.¹ Histological²⁻⁵ and histochemical⁶ studies on the role of vitamin A in vaginal keratinization lend support to the contention that vitamin A antagonizes the effect of estrogen *in vivo*. Furthermore, observations on integument *in vivo*⁷⁻¹⁴ and *in vitro*¹⁵⁻¹⁸ emphasize the role of vitamin A in epithelial differentiation.

In an attempt to determine specific hormone and vitamin actions, the technique of organ culture has been used. Organ culture¹⁹ involves the cultivation of fragments of organs or entire embryonic organs in a tissue-culture situation. Ultimately, one attempts to deal *in vitro* with normal tissue relationships as these exist in the body, divorced from the complexities of organ interaction.

Method and Materials

A modification of the watch-glass method²⁰ as devised by Chen²¹ and Shafer²² was utilized so that cultures were grown on a synthetic (chemically defined) medium, namely, CMRL 1066.† The method entails the use of a closed candle chamber to produce a carbon dioxide atmosphere sufficient (approximately 5 per cent) to maintain the pH of the medium at 7.2 to 7.4 (FIGURE 1). The cultures, derived from 3- to 4-week-old Sprague-Dawley rats, were explanted on either rayon acetate‡ or tantalum gauze§ and incubated at 37° C. from 1 to 6 days, having been subcultured every second day. Estradiol-17β|| and crystalline vitamin-A alcohol¶ were dissolved in absolute ethyl alcohol and serially diluted in the medium. The final concentration per watch glass was approximately 0.08 µg. of estradiol-17β (0.02 mg./100 ml. medium), approximately 0.3 µg. of crystalline vitamin-A alcohol (0.09 mg./100 ml. medium), and a 0.1 per cent ethanol concentration. The same amount of ethanol was added to control cultures. Samples were fixed at daily intervals in formal-acetic-alcohol and stained by the Regaud's iron hematoxylin Masson stain.

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† Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

‡ Voile, Silene, V. V. and Co., obtained through Robert Sayle and Co., Cambridge, England, and treated with Siliclad (Clay-Adams).

§ Ethicon, Inc., Type 50, Mesh 0.003 in., Code TM-53.

¶ Nutritional Biochemical Corp., Cleveland, Ohio, Lot #4820.

|| Kindly supplied by R. E. Kent, Chas. Püzer & Co., Inc., Groton, Conn.

Results

Prior to cultivation, the rodent vaginal epithelium appears as a stratified cuboidal epithelium varying in height from 5 to 10 cells. On occasion, the epithelium demonstrates evidence of an underlying squamous layer, possibly due to endogenous estrogens (FIGURE 2). Upon cultivation in the synthetic medium alone, the vaginal epithelium appears after 24 hours as a typical stratified squamous epithelium (FIGURE 3). After 48 hours *in vitro*, however, the epithelium begins to demonstrate keratinization of the underlying cells with progressive sloughing of the superficial cells (FIGURE 4). The addition of 0.08 μg . of estradiol-17 β per watch glass to the medium induces the precocious

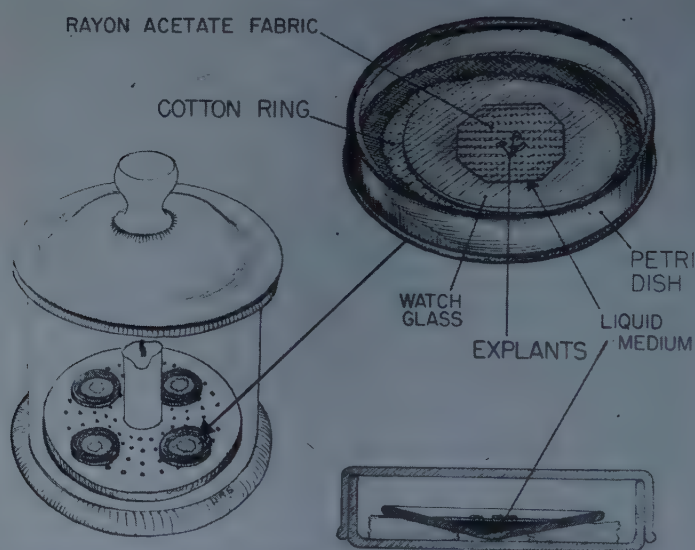


FIGURE 1.

development of keratinization after only 24 hours *in vitro* (FIGURE 5). Continued cultivation results in a greater degree of keratinization with concomitant loss of the thickness of the epithelium (FIGURE 6). The addition of vitamin-A alcohol to the medium (0.3 μg . per watch glass) results in a somewhat different situation. After 24 hours the epithelium continues to demonstrate the cuboidal nature of the superficial layer (FIGURE 7) seen in the controls, and remains so after 48 hours (FIGURE 8), but by the third day (72 hours) the epithelium again demonstrates the presence of keratinization (FIGURE 9).

Discussion

Whereas Emmens and Ludford²³ were unsuccessful in their attempt to grow rodent vagina *in vitro*, Hardy *et al.*²⁴ reported their success in growing fetal mouse vagina by the hanging-drop method, using a natural medium; they



FIGURE 2. Section of vaginal mucosa of 3-week-old rat, prior to cultivation. Note the squamous cells that are seen on occasion beneath the superficial cuboidal layer. $\times 650$.

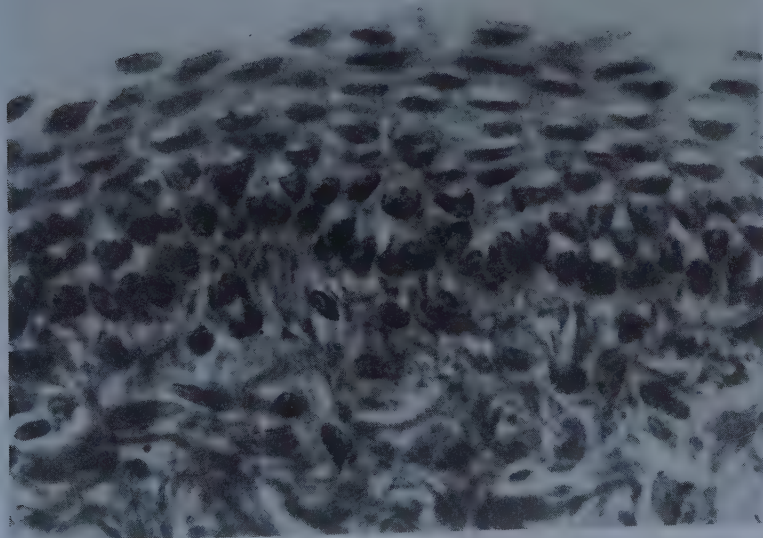


FIGURE 3. Section of vaginal mucosa of 3-week-old rat, cultured for 1 day in synthetic medium 1066. The epithelium is not keratinized. $\times 650$.



FIGURE 4. Section of vaginal mucosa of 3-week-old rat, cultured for 2 days in synthetic medium 1066. Keratinization present. $\times 650$.

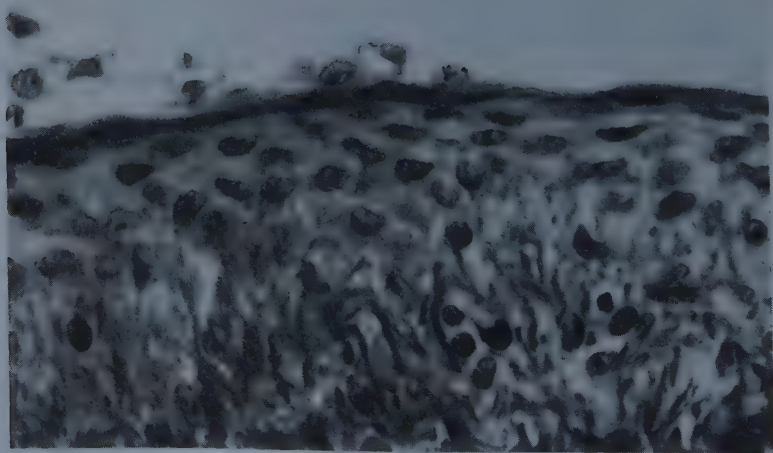


FIGURE 5. Section of vaginal mucosa of 3-week-old rat, cultured for 1 day in synthetic medium 1066 plus $0.08 \mu\text{g.}$ of estradiol- 17β . Note precocious keratinization. $\times 650$.

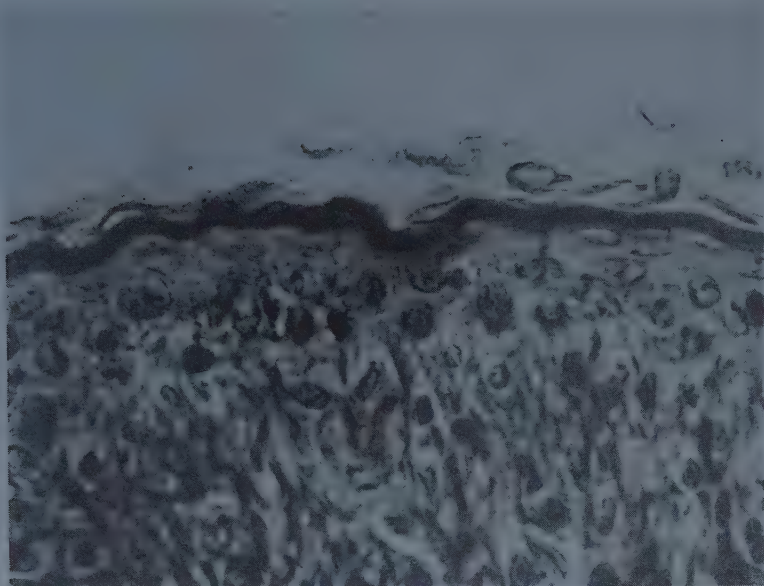


FIGURE 6. Section of vaginal mucosa of 3-week-old rat, cultured for 2 days in synthetic medium 1066 plus 0.08 μ g. of estradiol-17 β . $\times 650$.



FIGURE 7. Section of vaginal mucosa of 3-week-old rat, cultured for 1 day in synthetic medium 1066 plus 0.03 μ g. of crystalline vitamin-A alcohol. $\times 650$.

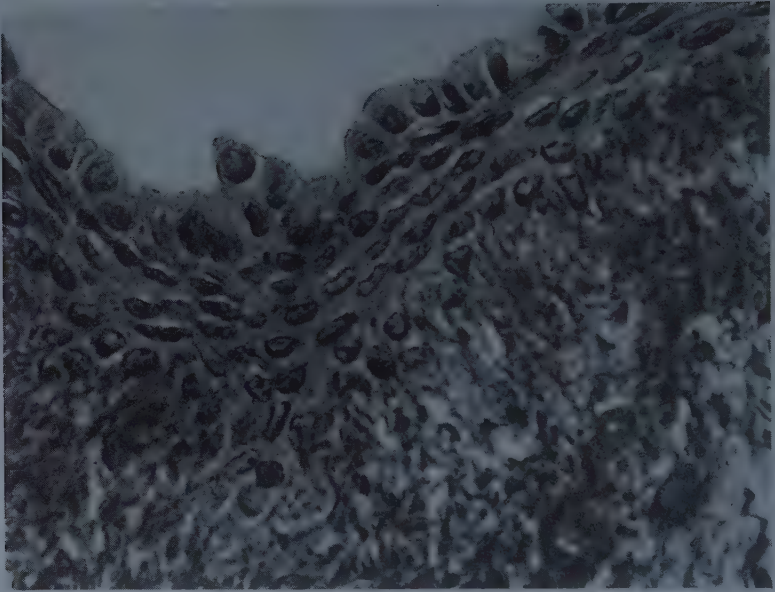


FIGURE 8. Section of vaginal mucosa of 3-week-old rat, cultured for 2 days in synthetic medium 1066 plus $0.03\text{ }\mu\text{g.}$ of crystalline vitamin-A alcohol. $\times 650$.



FIGURE 9. Section of vaginal mucosa of 3-week-old rat, cultured for 3 days in synthetic medium 1066 plus $0.03\text{ }\mu\text{g.}$ of crystalline vitamin-A alcohol. Keratinization present. $\times 650$.

demonstrated that direct application of estrogens to the isolated vagina resulted in its keratinization, inferring therefrom that estrogens can act directly and are not metabolized systemically, as suggested by Szego and Roberts.²⁵ We were similarly successful in the cultivation of the prepuberal rat vaginal epithelium, using the watch-glass method and a purely synthetic medium. Keratinization has been produced precociously by small amounts of estradiol-17 β added to the medium. Nevertheless, the local response to a steroid under the simplified conditions of organ culture may not be evoked in the same way as a systemically distributed folliculoid. Thus, these *in vitro* results in themselves do not exclude the possibility of metabolic activation of the steroid, as suggested by Szego and Roberts.²⁵ It should be pointed out that the vaginal explants grown on the synthetic medium alone, used as controls, also underwent keratinization, although not as rapidly as those treated with the steroid. This keratinization of the controls can not be attributed, as previously suggested when natural media (embryonic extract and plasma) were used,⁴ to the presence of a factor(s) in the synthetic medium that specifically induces the phenomenon. It would appear that either the ability to keratinize is inherent in this tissue, as inferred by Moore,²⁶ or the medium and conditions used in this investigation lack some specific requirement(s). Moore points out that the vaginal epithelium in an unstimulated state *in vivo* undergoes a type of cornification. The fact that vitamin A delays the onset of keratinization of the vaginal epithelium *in vitro* is indicative, although not conclusive, of its direct action to inhibit this phenomenon. Possibly other physical factors, such as oxygen tension,²⁷ may be required to act synergistically with the vitamin on this tissue *in vitro* in order to corroborate the previous *in vivo* findings.²⁻⁶

Fell and Mellanby¹⁵ demonstrated that the addition of vitamin A to organ cultures of embryonic chick ectoderm not only inhibited keratinization but caused its differentiation into a mucus-secreting type. Weiss and James^{17, 18} reported that only a brief exposure to the vitamin was necessary to inhibit keratinization of the embryonic chick ectoderm for a period of at least five days *in vitro*. They concluded that the vitamin was acting as a reversible inductor. Topically applying extremely small amounts of vitamin A to the skin of young adult mice, Lawrence and Bern¹⁴ were able to induce epidermal hypertrophy but could not establish any inhibition of keratinization.

It can be concluded only that the role of vitamin A in epithelial differentiation still remains obscure. However, the technique of organ culture as recently improved by Trowell²⁷ provides a model system for further investigation of hormonal and nutritional actions.

Summary

A technique for the maintenance of the rodent vagina as an organized entity on a completely defined medium is described. Vaginal explants from prepuberal rats have been cultivated for from 1 to 6 days on a chemically defined synthetic medium (Connaught Medical Research Laboratories Medium #1066) using the watch-glass technique. Vaginal epithelium in the medium alone keratinizes after 2 days *in vitro*. Addition of approximately 0.08 μ g. of estradiol-17 β per watch glass induced the precocious development of a cornified

epithelium in 1 day. The addition of vitamin A (0.3 μ g. per watch glass) inhibits, but does not prevent, keratinization *in vitro*. The implications of these findings are discussed briefly.

Acknowledgment

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CONCLUDING REMARKS

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The process of summing up a monograph of the type recorded in the foregoing pages frequently is allied with mixed emotions. One is faced with what has been achieved and learned, on the one hand, and what is unsolved and needs clarification, on the other. I am sure, however, that everyone admits and welcomes the challenge of incompletely answered problems.

As pointed out by L. J. Wells, the upper four fifths of the vagina arise from a fusion of the Müllerian ducts; the lower one fifth, from the urogenital sinus portion of the cloaca. This latter portion is often involved in the anomalies of pseudohermaphroditism. The definitive vaginal epithelium is thought to be derived from epithelial cells that have migrated from the urogenital sinus. It is probable, too, that the maternal hormones of pregnancy, rather than the fetal gonads, directly govern the formation of the vagina. Certainly, the rapid decrease of the former hormones after delivery is followed by neonatal vaginal growth regression.

K. Krantz has made a special point of the intimacy and adherency of the lower two thirds of the urethra and the anterior vaginal wall, and has entered into the current controversy of vaginal "fascia" by stating that the vagina is surrounded by a thin plane of loose connective tissue rather than by a fascia in the accepted sense of the term. The histopathology of vaginal lesions, as seen on a surgical service, has been well outlined and discussed by C. M. Dougherty.

Vaginal fluid is a mixture of many elements including genital tract secretions, blood elements, and cellular debris, in addition to bacterial and viral flora. It is not surprising that enzymes might be found in such a fluid. W. H. Fishman and G. W. Mitchell have been greatly interested for some time in β -glucuronidase both in physiological states (the menstrual cycle, pregnancy, the menopause) and in pathological states (inflammations, including trichomonal vaginitis and carcinoma). Histochemical localization of this and other enzymes in the vaginal epithelium are recorded with reference to time of the menstrual cycle.

A. T. Gregoire and his associates have reported on the use of paper chromatography in separating vaginal fluid constituents, and have dealt specifically with amino acids. This particular method of separation should, incidentally, find wide application in studying other biological fluids, as well. L. Parsons and his co-workers have reinvestigated abdominovaginal electric potential differences with a modified, more sensitive instrument than used previously, and have demonstrated a relationship of electric potentials to ovarian function and ovulation timing. According to Parsons' studies, ovulation might occur much earlier than a fortnight before menstruation. E. Odeblad, a gynecologist and physicist, has presented his data on proton magnetic resonance with implications on intracellular and extracellular water of vaginal cells.

C. G. Rosa and J. T. Velardo have studied two oxidative enzyme systems,

succinic dehydrogenase (SDH) and diphosphopyridine nucleotide diaphorase (DPN), by histochemical means. Progesterone, when given to ovariectomized rats, demonstrated little effect in restoring vaginal SDH, while DPN was strongly affected at certain optimal concentrations. Estradiol tended to increase both SDH and DPN in areas of vaginal epithelium not undergoing cornification. Vaginal mucification is apparently related in some way to the production of mucopolysaccharides and DPN activity.

It is universally agreed that estrogen is the prime motivator of vaginal epithelial proliferation and differentiation. W. U. Gardner has stressed that in ovariectomized mice there are vaginal strain differences in response to estrogen when the latter is given either systemically or intravaginally, and that early environmental hormonal influences may modify greatly the subsequent response of the vagina to estrogen. R. A. Edgren, working with rats, has described, among other things, the effect of estrogen antagonists such as testosterone propionate. G. L. Wied has discussed the synergistic and antagonistic effects of androgens, estrogens, and progestogens as reflected in the human vaginal smear, and has related these effects to the existence of epithelial atrophy or proliferation. Vitamin A deficiency also causes vaginal keratinization and, *in vitro*, R. H. Kahn has demonstrated inhibition of keratinization with Vitamin A in explanted prepuberal rat vaginas previously treated with estrogen. This may be of clinical significance.

Vaginal bacteriology is a well-recognized factor in vaginal function and Döderlein's bacillus generally has been accepted as an indicator of vaginal well-being. However, Döderlein's bacillus, as demonstrated by C. A. Hunter and K. R. Long, is not one specific organism but, rather, a group of various strains. The bacteriology of the premenarchal vagina has been sporadically investigated. According to J. W. Huffman, the premenarchal vagina is relatively free of pathogens; fecal organisms are often found in premenarchal vaginal infections. The nonspore-forming anaerobes have been discussed by C. P. Jones and his co-workers; as might be anticipated, the significance of these microorganisms is not appreciated sufficiently, mainly because of infrequently utilized culture techniques. H. C. Hesseltine has reviewed the background of vaginal trichomonal and mycotic infections at various age periods. R. Wellerson and his associates have described metabolic studies on *Trichomonas vaginalis*, an organism producing a chronic, recurrent, and resistant inflammation that still defies successful management. Perhaps by knowing the metabolic activities of the trichomonad, effective therapy may eventually be possible. F. B. Carter has outlined the genera and species of fungi important in vaginal disease. *Hemophilus vaginalis* vaginitis is still a disputed clinical entity, accepted by H. L. Gardner and C. D. Dukes, but rejected by A. Heltai. A serious omission from the discussion has been, of course, the viral inhabitants of the vagina.

J. B. and R. M. Graham have published extensively on the important subject of vaginal cellular immunological response as manifested in normal vaginal parabasal cells, and we are privileged to have this subject reviewed and clarified. How specific the cellular changes are is yet to be determined. One of the fascinating sidelights of vaginal physiology is that of vaginal permeability; C. G. Hartman has presented an outline of the implications of the vaginal

membrane in this regard, and has stressed the unidirectional character of vaginal permeability.

The vagina is important in problems of fertility, although its role is less significant than, for example, the roles of the tubes or ovaries. The influence of vaginal infections and acidity has been summarized by E. T. Tyler. Related is the problem of vaginal lubrication at coitus which has been described by W. H. Masters. Of great interest is the finding that natural coital lubrication is mainly vaginal in origin and not cervical or Bartholinian, as frequently stated.

The scientific study of the vagina has been neglected for too long. As with all branches of medicine, there is yet considerable work to be done. We hope the foregoing papers will serve as guideposts for general methods and for directions of approach.

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